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Improving oocyte competence in dairy cows exposed to heat stress

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Project award year: 2014
Three year research project

ABSTRACT

Original Objectives. The overall goal is to develop methods to increase pregnancy rate in lactating dairy cows exposed to heat stress through methods that minimize damage to the oocyte and embryo caused by heat stress. Objectives were as follows: (1) examine the protective effects of melatonin on developmental competence of oocytes exposed to elevated temperature in vitro; (2) test whether melatonin feeding can improve developmental competence of oocytes in vivo and, if so, whether effects are limited to the summer or also occur in the absence of heat stress; and (3) evaluate the effectiveness of improving fertility by facilitating follicular turnover in the summer and winter.

Revised Objectives. **(**1) Examine protective effects of melatonin and follicular fluid on developmental competence of oocytes exposed to elevated temperature in vitro; (2) examine the protective effects of melatonin on developmental competence of embryos exposed to elevated temperature in vitro; (3) evaluate effectiveness of improving fertility by administering human chorionic gonadotropin (hCG) to increase circulating concentrations of progesterone and evaluate whether response to hCG depends upon genotype for four mutations reported to be related to cow fertility; and (4) identify genes with allelic variants that increase resistance of embryos to heat shock.

Background. The overall hypothesis is that pregnancy success is reduced by heat stress because of damage to the oocyte and cleavage-stage embryo mediated by reactive oxygen species (ROS), and that fertility can be improved by provision of antioxidants or by removing follicles containing oocytes damaged by heat stress. During the study, additional evidence from the literature indicated the potential importance of treatment with chorionic gonadotropin to increase fertility of heat-stressed cows and results from other studies in our laboratories implicated genotype as an important determinant of cow fertility. Thus, the project was expanded to evaluate hCG treatment and to identify whether fertility response to hCG depended upon single nucleotide polymorphisms (SNP) in genes implicated as important for cow fertility. We also evaluated whether a SNP in a gene important for cellular resistance to heat stress (*HSPA1L*, a member of the heat shock protein 70 family) is important for embryonic resistance to elevated temperature.

Major conclusions, solutions & achievements. Results confirmed that elevated temperature increases ROS production by the oocyte and embryo and that melatonin decreases ROS. Melatonin reduced, but did not completely block, damaging effects of heat shock on the oocyte and had no effect on development of the embryo. Melatonin was protective to the oocyte at 0.1-1 μM, a concentration too high to be achieved in cows. It was concluded that melatonin is unlikely to be a useful molecule for increasing fertility of heat-stressed cows. Treatment with hCG at day 5 after breeding increased first-service pregnancy rate for primiparous cows but not for multiparous cows. Thus, hCG could be useful for increasing fertility in first-parity cows. The effectiveness of hCG depended upon genotype for a SNP in *COQ9*, a gene encoding for a mitochondrial-function protein. This result points the way to future efforts to use genetic information to identify populations of cows for which hormone treatments will be effective or ineffective. The SNP in *HSPA1L* was related to embryonic survival after heat shock. Perhaps, genetic selection for mutations that increase cellular resistance to heat shock could be employed to reduce effects of heat stress on fertility.

Implications, both scientific and agricultural. This project has resulted in abandonment of one possible approach to improve fertility of the heat-stressed cow (melatonin therapy) while also leading to a method for improving fertility of primiparous cows exposed to heat stress (hCG treatment) that can be implemented on farms today. Genetic studies have pointed the way to using genetic information to 1) tailor hormonal treatments to cow populations likely to respond favorably and 2) select animals whose embryos have superior resistance to elevated body temperatures.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Book Chapter	0	0	1
Reviewed	0	1	1
Submitted	0	1	1

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
M.Sc. Student	Shik-Lang	Yael	Hebrew University	Israel
Ph.D. Student	Ortega	Sofia	University of Florida	USA
Ph.D. Student	Nathalia	Rocha	University of Florida	USA
M.Sc. Student	Grosman	Sofia	Hebrew University of Jerusalem	Israel
M.Sc. Student	Ortiz	William	University of Florida	USA
Ph.D. Student	Estrada-Cortes	Eliab	University of Florida	USA
Ph.D. Student	Sosa	Froylan	Cornell University	USA
Ph.D. Student	Rodriques	Thais	University of Florida	USA
Postdoctoral Fellow	Tuna	Kubra	University of Florida	USA
M.Sc. Student	Sacchenco	Ilya	Hebrew University	Israel
Ph.D. Student	Cavallari de Castro	Fernanda	University of Florida	USA
M.Sc. Student	Simoni	Chen	Hebrew University	Israel

Contribution of Collaboration

This project represents a synergistic collaboration between two research groups who each bring unique resources to bear on an agricultural problem of importance to both Israel and the United States. Drs. Hansen and Roth have jointly been performing research on improving fertility in heat-stressed cows since 2002. Their collaboration has been based on shared interests, friendship, and perceived opportunities for doing joint research that allows both to benefit from the unique resources available to each separately. Dr. Hansen has an excellent relationship with the dairy industry in Florida and south Georgia and has the capability of using commercial dairies for performing large-scale embryo transfer studies. Dr. Roth is skilled in the transvaginal, ultrasound-guided aspiration of follicles. The experiments involved a course of research that is integrated between both research laboratories. For example, experiments for Objective 1 were performed in both laboratories. There has been frequent communication between laboratories.

ACHIEVEMENTS

Effectiveness of melatonin for protecting the oocyte and embryo (Revised Obj. 1 and 2)

Experiments in both Israel and the USA evaluated thermoprotective effects of melatonin. For both the oocyte and zygote, culture at elevated temperature (41.0 or 41.5°C for oocytes and 40°C for zygotes) increased ROS production and 0.1-1.0 μM melatonin blocked this effect (see Figure 1 for representative effects). Melatonin (1.0 μM) improved developmental competence of oocytes that were either matured at 41°C for 14 h or exposed to the pro-oxidant menadione. In particular, melatonin prevented negative effects of heat shock or menadione on the proportion of oocytes that cleaved after fertilization and the proportion of cleaved embryos that developed to the blastocyst stage. When the incubation temperature was raised to 41.5°C and duration of heat shock was increased to 22 h, 0.1 μM melatonin was less able to maintain developmental competence. Melatonin increased the proportion of heat-shocked oocytes that completed nuclear maturation (as measured by the proportion of oocytes at meiosis II) but did not block the effect of heat shock on proportion of matured oocytes that cleaved after fertilization or that subsequently developed to the blastocyst stage (see Figure 2).

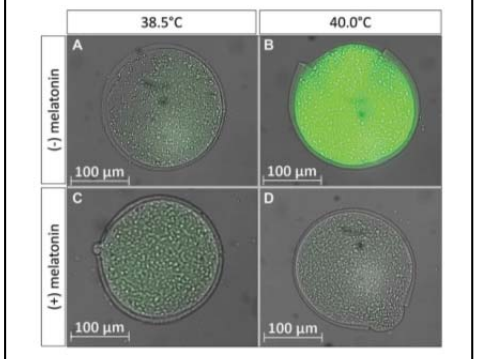

Figure 1. Representative images of zygotes labeled with CellROX to assess production of ROS.

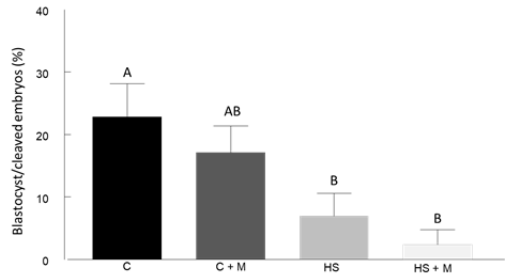

Figure 2. Effect of 0.1 μM melatonin (M) during oocyte maturation on competence of embryos derived from control (C) or heat-shocked (HS) oocytes to develop to the blastocyst stage. The HS was 41.5°C for 22 h. Data are means ± SEM. Different letters indicate significant differences at $P<0.05$.

The 1-cell embryo (zygote) is very sensitive to heat shock – exposure to temperatures as low as 40°C are sufficient to arrest development. We found that addition of 1.0 μM melatonin to culture medium of zygotes exposed to 40.0°C for 12 h did not block the negative effect of heat shock on development (not shown).

It should be noted that the concentrations of melatonin that were effective in reducing effects of 41.0°C on developmental competence of oocytes (1.0 μM) were much higher than could reasonably be achieved through melatonin administration by injection, implants, or feeding. Indeed, concentrations of melatonin in the blood of cows treated with 12 melatonin implants was in the

picomolar range (Garcia-Ispierto et al., Reprod Domest Anim 2013;48:577-583). Additional experiments from our laboratories indicated that treatment of oocytes with melatonin at concentrations less than 1.0 μM were without effect on oocyte developmental competence. Based on these results, the decision was made to abandon efforts to use melatonin to improve fertility.

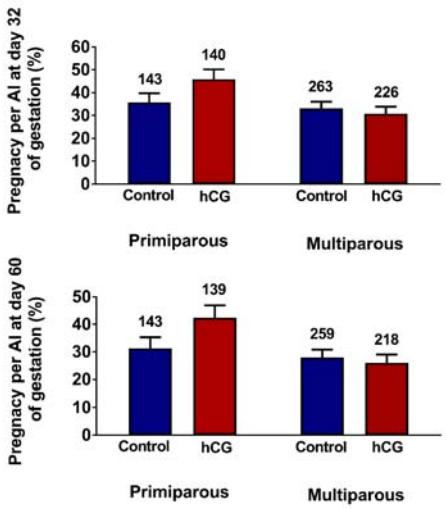

Figure 3. Effect of hCG on fertility. Values are least-squares means ± SEM of pregnancy per AI after TAI. Effect of hCG treatment on pregnancy per AI of primiparous (top) and multiparous cows (bottom). There was a tendency for a treatment x parity interaction on d 32 (P=0.098) and d 60 (P=0.063) of gestation, with hCG increasing pregnancy per AI in primiparous cows but not in multiparous cows.

We have also initiated experiments to determine whether follicular fluid contains molecules that can protect the oocyte from heat shock. Results indicate that exosomes in follicular fluid can reduce effects of heat shock on the maturing oocyte. This work may lead to new methods for protecting oocytes from heat shock.

Interactions between hCG and genotype on fertility during heat stress (Rev. Obj. 3)

Treatment with hCG at day 5 after artificial insemination (AI) has been reported to improve pregnancy per AI in lactating cows but there is much variation in treatment effectiveness (Nascimento et al., J. Dairy Sci. 2013; 96:2873–2882.). Season of the year may be an important determinant of effectiveness of hCG because heat stress can decrease circulating concentrations of progesterone (Howell et al., J. Dairy Sci. 1994; 77:735–739) and treatment with the related molecule, equine chorionic gonadotropin, at day 5 after AI improved pregnancy per AI in lactating cows exposed to heat stress (Garcia-Ispierto et al., Reprod Domest Anim 2013;48:577-83). For Objective 3, we tested whether fertility response to hCG in lactating Holsteins cows interacts with genotype and parity. Primiparous (n=538) and multiparous (n=613) cows were treated with hCG (3,300 IU) or vehicle 5 d after AI. Pregnancy was diagnosed on days 32 and 60 after artificial insemination (AI). A subset of cows (n=593-701) were genotyped for four SNP previously associated with fertility. Treatment with hCG increased progesterone concentration on day 12 after AI regardless of genotype or parity. Treatment with hCG tended to increase pregnancy per AI in primiparous cows but not in multiparous cows (Figure 3).

Moreover, hCG treatment interacted with a SNP in *COQ9* to affect fertility (Figure 4). Treatment with hCG increased pregnancy rate in AG cows (heterozygotes), decreased pregnancy rate in AA

cows and had no effect in GG cows. Stated differently, among cows treated with vehicle, AA cows had the highest pregnancy per AI at d 32 and 60 of gestation wherease, among hCG-treated cows, pregnancy per AI was lowest in AA cows. Pregnancy per AI was also affected by genotype for *HSPA1L* and *PGR*, but there were no interactions with treatment. Genotype for a SNP in *PARM1* was not associated with fertility. Overall, results show that variation in response to hCG treatment on fertility depends on parity and interacts with a SNP in *COQ9*.

Identify genes that increase resistance of embryos to heat shock (Revised Objective 4).

As illustrated in results from the previous experiment, the SNP in *HSPA1L* is important for dairy cow fertility. This SNP, located in the promoter, controls the expression level of the gene, which encodes for a heat shock protein important for cell survival to stress. To test whether this SNP affects embryonic resistance to heat shock, we genotyped blastocysts derived from zygotes exposed to 40°C for 12 h and compared the frequency of *HSPA1L* alleles to that of blastocysts derived from embryos not exposed to heat shock. Embryos were cultured in either low oxygen or high oxygen environments (high oxygen increases ROS production). The distribution of blastocysts according to the three genotypes (CC, CD (deletion), and DD) was affected by genotype x temperature x oxygen (P=0.0054). Frequency of the D allele (associated with higher expression of *HSPA1L*) was lower for blastocysts cultured at 38.5°C in low oxygen than for blastocysts produced under other conditions (not shown). This result suggests that inheritance of the D allele makes embryos better able to survive stressful conditions such as heat shock and a high oxygen environment.

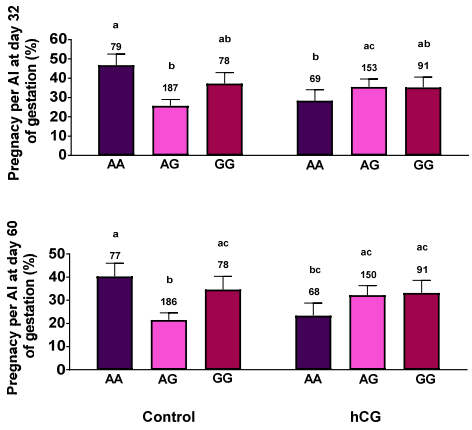

Figure 4. Effect of COQ9 genotype on the response to hCG treatment on pregnancy per AI. Values are least-squares means ± SEM of pregnancy per AI after TAI. There was a significant interaction between treatment with hCG and genotype for COQ9 on d 32 [P = 0.016 (top)] and d 60 [P = 0.012 (bottom)] of gestation. Bars with different superscripts differ (P<0.05).

Agricultural and Economic Impacts

This project has resulted in a method for improving fertility of primiparous cows exposed to heat stress (hCG treatment) that can be implemented on farms today. Genetic studies have pointed the way to using genetic information to 1) tailor hormonal treatments to cow populations likely to respond favorably and 2) select animals whose embryos have superior resistance to elevated body temperatures.

Changes to Original Research Plan

The overall goal is to develop methods to increase pregnancy rate in lactating dairy cows exposed to heat stress through methods that minimize damage to the oocyte and embryo caused by heat stress.

Original Objectives. (1) examine the protective effects of melatonin on developmental competence of oocytes exposed to elevated temperature in vitro; (2) test whether melatonin feeding can improve developmental competence of oocytes in vivo and, if so, whether effects are limited to the summer or also occur in the absence of heat stress; and (3) evaluate the effectiveness of improving fertility by facilitating follicular turnover in the summer and winter.

Revised Objectives. **(**1) Examine the protective effects of melatonin and follicular fluid on developmental competence of oocytes exposed to elevated temperature in vitro; (2) examine the protective effects of melatonin on developmental competence of embryos exposed to elevated temperature in vitro; (3) evaluate the effectiveness of improving fertility by administering human chorionic gonadotropin (hCG) to increase circulating concentrations of progesterone and evaluate whether response to hCG depends upon genotype for four mutations previously reported to be related to cow fertility; and (4) identify genes with allelic variants that increase resistance of embryos to heat shock.

Rationale. The overall hypothesis is that pregnancy success is reduced by heat stress because of damage to the oocyte and cleavage-stage embryo mediated by reactive oxygen species (ROS), and that fertility can be improved by provision of antioxidants or by removing follicles containing oocytes damaged by heat stress. During the study, additional evidence from the literature indicated the potential importance of treatment with chorionic gonadotropin to increase fertility of heat-stressed cows and results from other studies in our laboratories implicated genotype as an important determinant of cow fertility. Thus, the project was expanded to evaluate hCG treatment and to identify whether fertility response to hCG depended upon single nucleotide polymorphisms (SNP) in genes implicated as important for cow fertility. We also evaluated whether a SNP in a gene important for cellular resistance to heat stress (*HSPA1L*, a member of the heat shock protein 70 family) is important for embryonic resistance to elevated temperature. Finally, we conducted an experiment suggesting the existence of thermoprotective molecules in exosomes located in follicular fluid.

Publications for Project US-4709-14

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*Ortega, M.S., Rocha-Frigoni, N.A.S., Mingoti, G.Z., Roth, Z., and Hansen, P.J.*	Modification of embryonic resistance to heat shock in cattle by melatonin and genetic variation in HSPA1L	***Journal of Dairy Science***	99 : 9152-9164 2016	Joint
Published	Book Chapter	*Hansen, P.J.*	Physiological approaches to improving fertility during heat stress.	***Large Herd Dairy Management, 3rd Ed.***	: in press 2016	US only
Accepted	Reviewed	*Zolini, A.M., Ortiz, W.G., Estrada-Cortes, A., Ortega, M.S., Dikmen, S., Sosa, F., Giordano, J.O., and Hansen, P.J.*	Interactions of human chorionic gonadotropin with genotype and parity on fertility responses of lactating dairy cows.	***Journal of Dairy Science***	:	US only
Submitted	Reviewed	*Cavallari de Castro, F., Lima Verde Leal, C., Roth, Z., and Hansen, P.J.*	Effects of melatonin on production of reactive oxygen species and developmental competence of bovine oocytes exposed to heat shock and oxidative stress during in vitro maturation.	***Molecular Reproduction and Development***	:	Joint
Submitted	Reviewed	*Rodriques, T.A., Tuna, K.M., Alli, A.A., Tribulo, P., Hansen, P.J., Koh, J., and Paul-Lopes, F.F.*	Follicular fluid exosomes act on the bovine oocyte to improve oocyte competence to support development and survive heat shock.	***Reproduction Fertility and Development***	:	US only

J. Dairy Sci. 99:9152–9164
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Modification of embryonic resistance to heat shock in cattle by melatonin and genetic variation in *HSPA1L*

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ABSTRACT

The objectives were to test whether (1) melatonin blocks inhibition of embryonic development caused by heat shock at the zygote stage, and (2) the frequency of a thermoprotective allele for *HSPA1L* is increased in blastocysts formed from heat-shocked zygotes as compared with blastocysts from control zygotes. It was hypothesized that melatonin prevents effects of heat shock on development by reducing accumulation of reactive oxygen species (ROS) and that embryos inheriting the thermoprotective allele of *HSPA1L* would be more likely to survive heat shock. Effects of 1 μM melatonin on ROS were determined in experiments 1 and 2. Zygotes were cultured at 38.5 or 40°C for 3 h in the presence of CellROX reagent (ThermoFisher Scientific, Waltham, MA). Culture was in a low [5% (vol/vol)] oxygen (experiment 1) or low or high [21% (vol/vol)] oxygen environment (experiment 2). Heat shock and high oxygen increased ROS; melatonin decreased ROS. Development was assessed in experiments 3 and 4. In experiment 3, zygotes were cultured in low oxygen ± 1 μM melatonin and exposed to 38.5 or 40°C for 12 h (experiment 1) beginning 8 h after fertilization. Melatonin did not protect the embryo from heat shock. Experiment 4 was performed similarly except that temperature treatments (38.5 or 40°C, 24 h) were performed in a low or high oxygen environment (2 × 2 × 2 factorial design with temperature, melatonin, and oxygen concentration as main effects), and blastocysts were genotyped for a deletion (D) mutation (C→D) in the promoter region of *HSPA1L* associated with thermotolerance. Heat shock decreased percent of zygotes developing to the blastocyst stage independent of melatonin or oxygen concentration. Frequency of genotypes for *HSPA1L* was affected by oxygen concentration and temperature, with an increase in the D allele for blastocysts that developed in high oxygen and following heat shock. It was concluded that (1) lack of effect of melatonin or oxygen concentration on embryonic development means that the negative effects of heat shock on the zygote are not mediated by ROS, (2) previously reported effect of melatonin on fertility of heat-stressed cows might involve actions independent of the antioxidant properties of melatonin, and (3) the deletion mutation in the promoter of *HSPA1L* confers protection to the zygote from heat shock and high oxygen. Perhaps, embryonic survival during heat stress could be improved by selecting for thermotolerant genotypes.
Key words: heat shock, melatonin, reactive oxygen species, *HSPA1L*

INTRODUCTION

In lactating dairy cattle, environmental temperatures as low as 23 to 29°C can lead to hyperthermia (Sartori et al., 2002; Dikmen and Hansen, 2009). Increases in body temperature to around 39°C are associated with reduced pregnancy rates (Gwazdauskas et al., 1973) and therefore pregnancy rates per AI decline during period of heat stress (Hansen and Aréchiga, 1999; Flamenbaum and Galon, 2010). One of the actions of heat stress responsible for lower fertility are actions on oocyte maturation (Putney et al., 1989), fertilization (Sartori et al., 2002), and early embryonic development (Ealy et al., 1993). Embryos are particularly susceptible to the effects of heat stress in vivo and exposure to elevated temperature in vitro during early stages of development, from the 1-cell through 4-cell stages of development (Ealy et al., 1995; Edwards and Hansen, 1997; Sakatani et al., 2012).

One of the proposed mechanisms by which heat stress can damage the oocyte and the embryo is through pro-

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duction of reactive oxygen species (**ROS**). Exposure of the oocyte and embryo to elevated temperatures can increase ROS production (Sakatani et al., 2004; Nabenishi et al., 2012b). Some antioxidants have been reported to reduce effects of heat shock in cultured embryos including anthocyanin (Sakatani et al., 2007) and dithiothreitol (de Castro e Paula and Hansen, 2008), whereas others such as glutathione (Ealy et al., 1995) and vitamin E (Paula-Lopes et al., 2003a) had little effect on embryo susceptibility to heat shock. Recently, it has been reported that supplementation of melatonin to oocytes exposed to heat shock during the maturation period reduced negative effects of heat shock (Cebrian-Serrano et al., 2013). Moreover, administration of slow-release implants of melatonin to lactating cows exposed to heat stress improved fertility (Garcia-Ispierto et al., 2013b).

Genetic effects also occur on resistance of the embryo to heat shock, as is evidenced by the observation that heat shock reduced development of bovine embryos from thermotolerant breeds less than for less adapted breeds (Paula-Lopes et al., 2003b; Eberhardt et al., 2009; Silva et al., 2013). One family of genes involved in cellular thermoprotection is the heat shock protein 70 (**HSP70**) family (Christians et al., 2003). In the cow, 2 HSP70 genes exist: *HSPA1A* and *HSPA1L*. Reverse-transcription PCR using primers that do not distinguish between the 2 genes indicated that heat shock can increase transcription of *HSPA1A/A1L* as early as the 2-cell stage (Chandolia et al., 1999; Sakatani et al., 2012). A mutation in the promoter region of *HSPA1L* (Rosenkrans et al., 2010; Ortega et al., 2016) that results in a deletion of a cytosine (**C**) has been associated with thermotolerance in peripheral blood mononuclear cells exposed to heat stress (Basiricò et al., 2011). The same mutation has been associated with superior embryonic development to the blastocyst stage in cultured embryos (Cochran et al., 2013). It is possible, therefore, that inheritance of this allele can increase embryonic resistance to heat shock.

The objective of this study was to determine if the deleterious effects of exposure of bovine embryos to heat shock at the zygote stage of development is modified by melatonin or inheritance of the deletion (**D**) allele of *HSPA1L*. The effect of melatonin was tested by evaluating whether its addition to culture medium would reduce effects of heat shock on production of ROS and inhibition of embryonic development. The effect of *HSPA1L* genotype was determined indirectly by testing whether the allele frequency of the D allele (the putative thermotolerant allele) would be greater in blastocysts developing after exposure to heat shock than for blastocysts not exposed to heat shock.

MATERIALS AND METHODS

In Vitro Production of Embryos

Ovaries were obtained from Central Packing Co. (Center Hill, FL) from cattle of *Bos taurus* and various admixtures of *B. taurus* and *Bos indicus* breeds. The surface of each ovary was cut with a scalpel to harvest immature cumulus-oocyte complexes (**COC**) from follicles 2 to 8 mm in diameter into oocyte washing medium (BoviPRO), which contained salts, bicarbonate, HEPES, DL-lactic acid, and BSA and was purchased from MOFA Global (Verona, WI). The COC were washed and those having uniform cytoplasm and at least 3 layers of cumulus cells were matured in groups of 10 in 50-μL droplets of oocyte maturation medium (composition of all media is presented in Supplemental Table S1; http://dx.doi.org/10.3168/jds.2016-11501) covered with mineral oil for 21 h at 38.5°C in a humidified atmosphere of 5% (vol/vol) CO_2. For each replicate, up to 300 COC were matured. After maturation, COC were washed 3 times in HEPES-TALP (Tyrode's albumen lactate pyruvate) medium and placed in a 35-mm dish containing 1.7 mL of fertilization medium (IVF-TALP). Insemination of each replicate of fertilization was performed with semen pooled from 3 individual bulls of various taurine breeds (the total number of bulls were 29). Sperm were purified from frozen-thawed straws of extended semen using an Isolate gradient [Irvine Scientific, Santa Ana, CA; 50% (vol/vol) and 90% (vol/vol) isolate] and diluted in IVF-TALP to achieve a final concentration of 1×10^6/mL in the fertilization dish. In addition, 80 μL of penicillamine-hypotaurine-epinephrine solution was added to each fertilization well to improve sperm motility and promote fertilization. Fertilization proceeded for 8 to 9 h at 38.5°C in a humidified atmosphere of 5% (vol/vol) CO_2.

Putative zygotes (i.e., oocytes exposed to sperm) were denuded from the surrounding cumulus cells at the end of fertilization by vortexing groups of 200 to 300 putative zygotes for 5 min in 600 μL of HEPES-TALP containing 10,000 U/mL of hyaluronidase. Unless otherwise stated, embryos were cultured in groups of 25–30/50 μL microdrops of culture medium (SOF-BE2) prepared with or without 1 μ*M* melatonin (Santa Cruz Biotechnologies, Dallas, TX), which was dissolved directly in SOF-BE2. The microdrops were covered with mineral oil at 38.5°C in a humidified atmosphere of 5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2. When embryos were heat shocked, culture was performed under either high oxygen conditions [40.0°C in a humidified atmosphere of 6% (vol/vol) CO_2 and atmospheric oxygen] or low oxygen conditions [40.0°C

in a humidified atmosphere of 5% (vol/vol) O_2 and 6% (vol/vol) CO_2 with the balance as N_2]. The increase in CO_2 for heat shock was to account for its reduced solubility at a higher temperature. Heat shock was initiated 8 h after insemination (at the end of fertilization) and proceeded for 3, 12, or 24 h. Afterward, heat-shocked embryos were examined for ROS production (3 h) or were returned to the same environment as the embryos not exposed to heat shock [38.5, 5% (vol/vol) O_2 and 5% (vol/vol) CO_2 in a humidified incubator with the balance N_2].

Percentage of putative zygotes that cleaved was determined at Day 3 of development (Day 0 = day of fertilization) and the percentage of putative zygotes and cleaved embryos that became blastocysts was determined at Day 7 of development.

Detection of Reactive Oxygen Species

Production of ROS by embryos was determined by incubating putative zygotes for 3 h in SOF-BE2 containing 5 μM CellROX green reagent (ThermoFisher Scientific, Waltham, MA). This reagent is a cell-permeant dye that exhibits bright green photostable fluorescence upon oxidation and subsequent binding to DNA. After incubation with CellROX, embryos were washed 3 times in 50-μL droplets of Dulbecco's phosphate-buffered saline (**DPBS**) containing 1% (wt/vol) polyvinylpyrrolidone (**PVP**), fixed in 4% (wt/vol) paraformaldehyde in DPBS, washed 3 additional times in DPBS-PVP and mounted in groups of 10 embryos on microscope slides using Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA). Embryos were examined for fluorescence within 10 h after labeling by fluorescence microscopy using a green emission filter with a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Digital images of each embryo were acquired using AxioVision software (v. 4.8.2, Zeiss, Peabody, MA) and a high-resolution black and white Zeiss Axiocam MRM digital camera. Analysis of the images was performed using ImageJ V. 1.48 (National Institutes of Health, Bethesda, MD). Net fluorescent intensity was calculated by obtaining the average pixel intensity of each embryo (obtained after manually drawing a boundary around the embryo) and subtracting the background intensity obtained from a region of the image not containing the embryo.

Genotype Determination and Frequency

Day 7 blastocysts were genotyped for a SNP in the promoter region of *HSPA1L* (UMD3.1.1 chr 23:27334005) in which a C is replaced by a deletion (C/D mutation; Rosenkrans et al., 2010; Supplemental Figure S1; http://dx.doi.org/10.3168/jds.2016-11501). Blastocysts were washed 3 times in PBS-PVP and the zona pellucida denuded using 0.1% (wt/vol) protease from *Streptococcus griseus*. After further washing, embryos were placed individually in wells of 96-well plates with 5 μL of molecular biology–grade water for subsequent genotyping by PCR using an anchored KASP by design assay (LGC Genomics, Middlesex, UK). The assay is a PCR-based technique involving a common reverse primer and 2 allele-specific forward primers, with one bound to fluorescein amidite (**FAM**) and the second to hexachlorofluorescein (**HEX**) to produce genotype-specific PCR products. The primer sequences (5′ → 3′) to target each allele were CAAGTCCTGCCCCCTGC-FAM for the deletion allele and CTCAAGTCCT-GCCCCCTGG-HEX for the C allele. The common reverse primer was GCATCCAGGGCGCTGATTG-GTT. Each PCR reaction included 5 μL of embryo resuspended in molecular biology–grade water, 5 μL of 2× supermix with low ROX (LGC Genomics), and 0.14 μL of KASP by design primer mix. Amplification and analysis were performed using a CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA); thermal cycling conditions were 94°C for 15 min, followed by 10 cycles of 94°C for 20 s and 61°C for 60 s, where the second temperature was decreased 0.6°C per cycle to achieve a final annealing temperature of 55°C at the end of the 10th cycle. The reaction proceeded for an additional 26 cycles of 94°C for 20 s and 55°C for 60 s, and a read step of 37°C for 60 s. To improve genotype cluster visibility, 3 additional cycles of 94°C for 20 s and 55°C for 60 s and a final read step at 37°C for 60 s were performed. In each run, DNA samples from whole blood of cows of known genotypes were analyzed as controls. Determination of the genotype was performed using the allelic discrimination feature of the CFX96 machine. A representative output is shown in Figure 1.

Experiments

Experiment 1 was performed to determine (1) if exposure to heat shock increased ROS production in the embryo, and (2) whether melatonin blocked ROS production. The experiment was a 2 × 2 factorial design including 2 temperature treatments (38.5 and 40°C) and 2 melatonin concentrations (0 and 1 μM). Putative zygotes were produced from a single in vitro production of embryos (**IVP**) procedure involving embryos produced by fertilization with semen pooled from 3 bulls. Groups of putative zygotes supplemented with 0 or 1 μM melatonin were cultured during 3 h at either 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2], or 40°C [5% (vol/vol) O_2 and 6% (vol/vol) CO_2 with the balance as N_2] beginning 8 h after

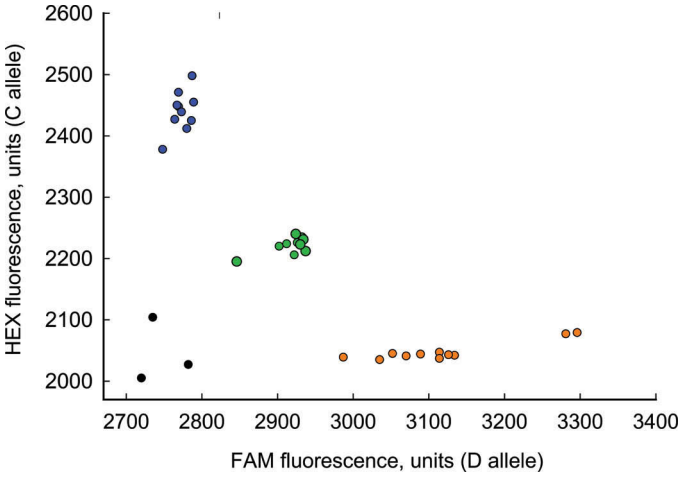

Figure 1. Representative results for genotyping assay for the deletion mutation in *HSPA1L*. Representative example of results from the genotype assay for the SNP in *HSPA1L* using the KASP assay (LGC Genomics, Middlesex, UK). In the assay, PCR amplicons from embryos with the cytosine (C) allele hybridize to the hexachlorofluorescein fluorprobe (HEX), whereas amplicons from embryos with the deletion (D) allele hybridize to the fluorescein amidite fluoroprobe (FAM). Embryos classified as homozygous for C are indicated by black (blue) circles, embryos homozygous for the deletion allele are indicated by light gray (orange) circles and heterozygotes are indicated by dark gray (green) circles. The 3 black circles at the bottom left of plot represent no signals (no genotype assigned). Color version available online.

insemination. After treatment, putative zygotes were assessed individually for ROS production as described above. The number of embryos evaluated varied from 91 to 117 per group.

Experiment 2 was conducted similarly to experiment 1 except the effect of oxygen concentration in the culture environment was also determined. The experiment involved a 2 × 2 × 2 factorial design including 2 temperature treatments (38.5 and 40°C), 2 melatonin concentrations (0 and 1 μM), and 2 oxygen environments (5 and 21% vol/vol O_2). Putative zygotes were produced from a single IVP procedure involving fertilization with sperm from a pool of 3 bulls. Beginning 8 h after insemination, putative zygotes supplemented with 0 or 1 μM melatonin were cultured for 3 h in a low oxygen environment at either 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2], or 40°C [5% (vol/vol) O_2 and 6% (vol/vol) CO_2 with the balance as N_2] or in a high oxygen environment at either 38.5 in a humidified atmosphere of 5% (vol/vol) CO_2 or at or 40°C in a humidified atmosphere of 5% (vol/vol) CO_2. The number of putative zygotes per treatment ranged from 20 to 36.

Experiment 3 was performed to determine if melatonin blocks the negative effects of heat shock at the zygote stage on embryonic development to the blastocyst stage. The experiment was a 2 × 2 factorial design which included 2 melatonin concentrations (0 and 1 μM) and 2 temperatures (38.5 or 40°C). Putative zygotes supplemented with 0 or 1 μM were cultured at either 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2], or 40°C [5% (vol/vol) O_2 and 6% (vol/vol) CO_2 with the balance as N_2]. Heat shock treatment started 8 h after insemination and lasted for 12 h, after which all groups were cultured at 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2] until Day 7 of development. Endpoints were percent of putative zygotes that cleaved byDay 3 of development, percent of putative zygotes that became a blastocyst at Day 7 of development, and percent of cleaved embryos that became a blastocyst at Day 7 of development. The experiment was conducted in 22 replicates using a total of 25 bulls for fertilization. The total number of putative zygotes per treatment ranged from 1,451 to 1,532.

Experiment 4 was designed to test interactions between melatonin, oxygen concentration, and temperature on embryonic development. The experiment had a 2 × 2 × 2 factorial design including 2 temperature treatments (38.5 and 40°C), 2 melatonin concentrations (0 and 1 μM), and 2 oxygen environments (5 and 21% vol/vol O_2). Beginning 8 h after insemination, putative zygotes supplemented with 0 or 1 μM melatonin were cultured for 24 h at either 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2], or 40°C [5% (vol/vol) O_2 and 6% (vol/vol) CO_2 with the balance as N_2] in a low oxygen (5% vol/vol) environment or at either 38.5 or 40°C in a humidified atmosphere of 5% (vol/vol) CO_2 in a high oxygen environment. Thereafter, all embryos were cultured at 38.5°C [5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2] until Day 7 of development. The experiment was performed in 8 replicates with a total of 19 sires and the number of putative zygotes ranged from 510 to 578 per treatment. Endpoints were as for experiment 3. In addition, 542 blastocysts from the experiment were collected individually and genotyped for the deletion mutation in *HSPA1L*, as described above.

Statistical Analysis

Differences in ROS intensity were evaluated by ANOVA using the GLM procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). For experiment 1, the model included temperature, melatonin treatment, and the interactions as fixed effects. For experiment 2, the model included temperature, melatonin treatment, oxygen, and their interactions as fixed effects. In both experiments 1 and 2, each embryo was analyzed individually and considered an experimental unit. In experiment 1, the total amount of experimental units

was 416. In experiment 2, the total amount of experimental units was 205.

For experiments 3 and 4, data on percent cleavage, putative zygotes, and cleaved embryos becoming blastocysts calculated for each replicate were analyzed by ANOVA using the MIXED procedure of SAS. The main effects included in the model were temperature, melatonin, and their interactions for experiment 3; and temperature, melatonin, oxygen, and their interactions for experiment 4. In both cases, replicate was included as random effect in the model.

To determine effect of treatment on blastocyst genotype, the frequency of each genotype (CC, CD, DD) was calculated for each replicate. Treatment effects on frequency were determined by the ANOVA with the GLIMMIX procedure, using a Poisson distribution. Frequency was the response variable and the model included genotype, temperature, oxygen, and their interactions as fixed effects, and replicate as a random effect.

RESULTS

ROS Production

In experiment 1, embryos were cultured in low oxygen. Representative images of ROS labeling are shown in Figure 2. When melatonin was added to culture medium, ROS intensity was reduced at both temperatures and embryos at 40°C did not have greater labeling intensity than embryos at 38.5°C (compare Figure 2C and Figure 2D).

Least squares means for ROS labeling intensity are shown in Figure 3. Significant effects were found for temperature ($P = 0.0012$), melatonin ($P = 0.0031$), and the temperature × melatonin interaction ($P <$

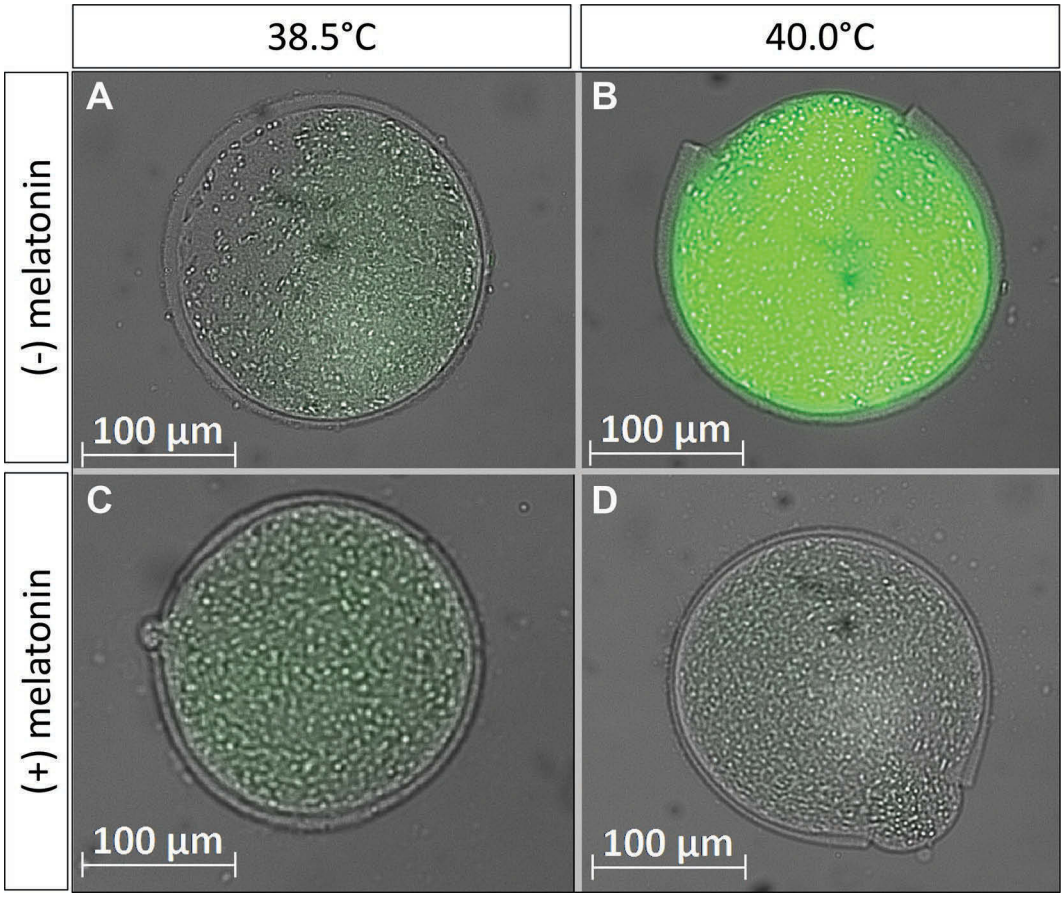

Figure 2. Representative images of zygotes labeled with CellROX (ThermoFisher Scientific, Waltham, MA) to assess production of reactive oxygen species as affected by incubation temperature and melatonin (experiment 1). Images were obtained by merging visualization of the embryo using differential interference and with a fluorescein isothiocyanate (FITC) fluorescent filter. Panels A and B represent embryos cultured without melatonin at 38.5°C (A) and 40°C (B). Panels C and D represent embryos cultured with melatonin at 38.5°C (C) and 40°C (D). Color version available online.

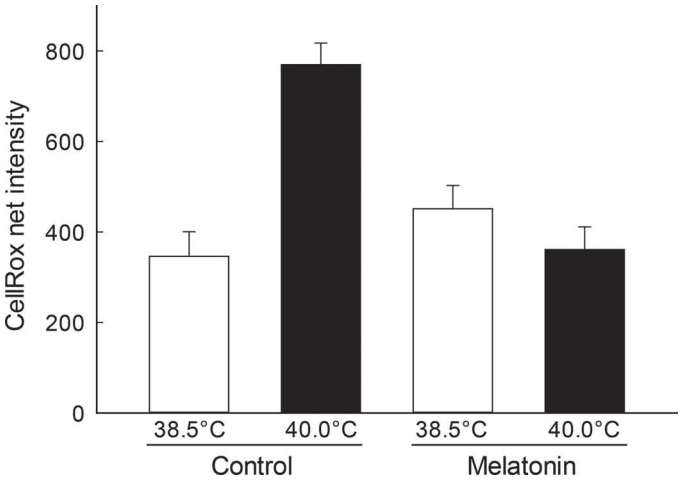

Figure 3. Effect of incubation temperature and melatonin on production of reactive oxygen species in the zygote as determined by CellROX (ThermoFisher Scientific, Waltham, MA) net intensity (experiment 1). Data are LSM ± SEM of arbitrary units of intensity. Intensity was affected by temperature ($P = 0.0012$), melatonin ($P = 0.0031$), and the temperature by melatonin interaction ($P < 0.0001$).

0.0001). These effects reflected the fact that heat shock increased labeling intensity and that melatonin reduced labeling intensity at both temperatures and prevented the increase in ROS labeling caused by culture at 40°C.

Experiment 2 was conducted similarly except that the experiment was performed under 2 gaseous atmospheres, high oxygen (21% O_2) and low oxygen (5% O_2) conditions. Representative images of ROS labeling are shown in Figure 4. Intensity of labeling was increased by culture at 40°C compared with 38.5°C for both oxygen environments (compare Figure 4A with 4B and 4E with 4F). Intensity was also greater for embryos in high oxygen than low oxygen (compare Figure 4A with 4E and 4B with Figure 4F). Melatonin decreased ROS production in embryos exposed to heat shock under low oxygen (compare Figure 4D with 4C) and high oxygen conditions (compare Figure 4H with 4G).

Least squares means for ROS intensity are shown in Figure 5. Intensity was affected by oxygen concentration ($P = 0.004$) and the interactions of temperature with melatonin treatment ($P = 0.0003$) and oxygen with melatonin treatment ($P = 0.0054$). These interactions occurred because melatonin prevented the increase in ROS intensity caused by culturing embryos in a high oxygen environment or at 40°C.

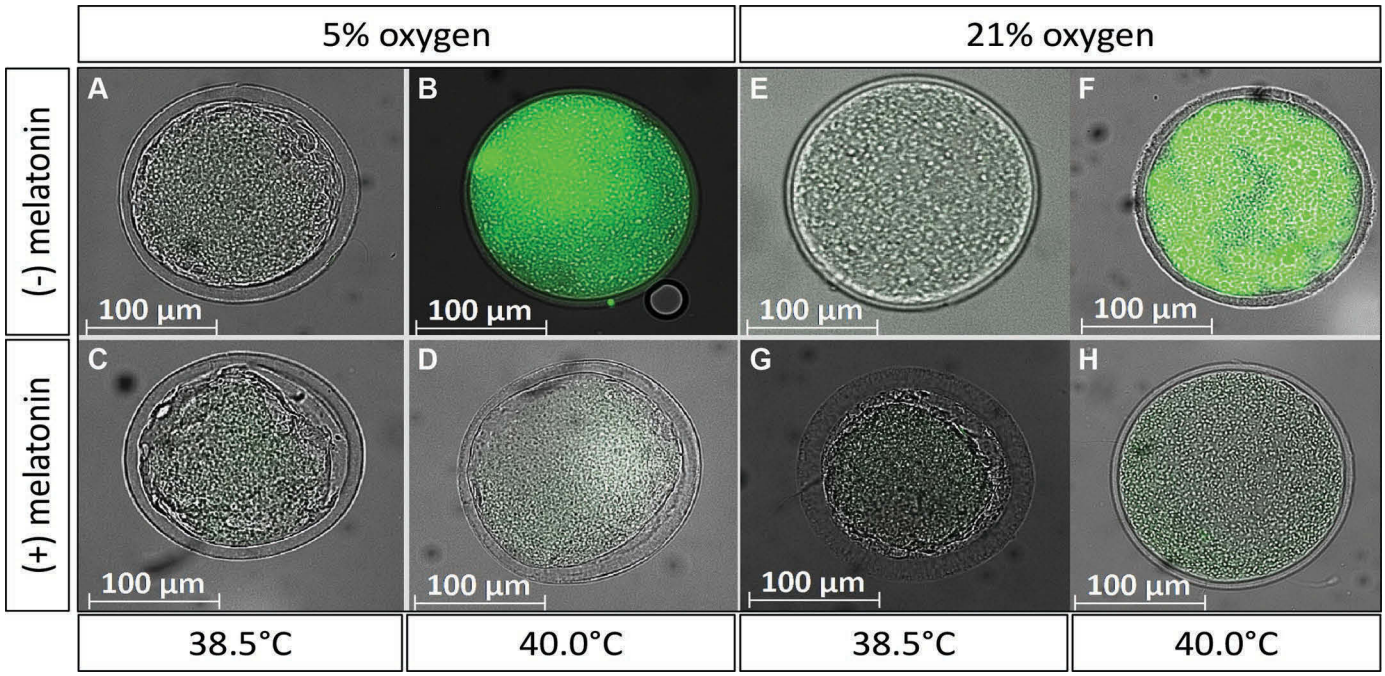

Figure 4. Representative images of zygotes labeled with CellROX (ThermoFisher Scientific, Waltham, MA) to assess production of reactive oxygen species as affected by incubation temperature and melatonin (experiment 2). Images were obtained by merging visualization of the embryo using differential interference and with a fluorescein isothiocyanate (FITC) fluorescent filter. Panels A to D represent embryos cultured in low oxygen [5% (vol/vol) O_2] for embryos cultured without melatonin at 38.5°C (A) and 40°C (B) and with melatonin at 38.5°C (C) and 40°C (D). Panels E to H represent embryos cultured in high oxygen [21% (vol/vol) O_2] without melatonin at 38.5°C (E) and 40°C (F) or with melatonin at 38.5°C (G) and 40°C (H). Color version available online.

Development to the Blastocyst Stage

For experiment 3, embryos were cultured continuously in low oxygen. Results are shown in Figure 6. Culture at 40°C during the first 12 h of culture decreased the percent of putative zygotes that cleaved ($P = 0.0012$) and that progressed to the blastocyst stage ($P < 0.0001$) as well as the percent of cleaved embryos that became blastocysts ($P < 0.0001$). No effect was found of melatonin or temperature by melatonin interactions on any developmental trait measured.

Experiment 4 was conducted similarly except that heat shock was for 24 h and embryos were cultured either in low oxygen continuously or were moved to high oxygen during the period corresponding to temperature treatments. Results are presented in Figure 7. No effect was found of temperature treatment, oxygen concentration, or the interaction on the percent of putative zygotes that cleaved, although culture at 40°C tended to reduce cleavage ($P = 0.09$). Elevated temperature also reduced the percent of putative zygotes that became blastocysts ($P = 0.003$) and the percent of cleaved embryos developing to blastocysts ($P = 0.01$). Neither oxygen, melatonin, nor interactions with these 2 main effects affected development to the blastocyst stage.

Genotype

The genotype of embryos that developed to the blastocyst stage was determined in experiment 4. As shown in Figure 8, the distribution of blastocysts according to the 3 genotypes (CC, CD, DD) was affected by temperature (genotype × temperature, $P < 0.0001$), oxygen environment (genotype × oxygen, $P < 0.0001$), and the interaction of temperature by oxygen (genotype × temperature × oxygen, $P = 0.0054$). These effects reflected the fact that frequency of the D allele was lower for blastocysts cultured at 38.5°C in low oxygen than for blastocysts produced under other conditions. For embryos cultured at 38.5°C in low oxygen (i.e., the least stressful environment for the embryo), the overall frequency of the D allele was 33%. In other groups, however, the frequency of the D allele was higher, ranging from 43 to 56%. No effect was found of melatonin or interactions of melatonin with other main effects on genotype.

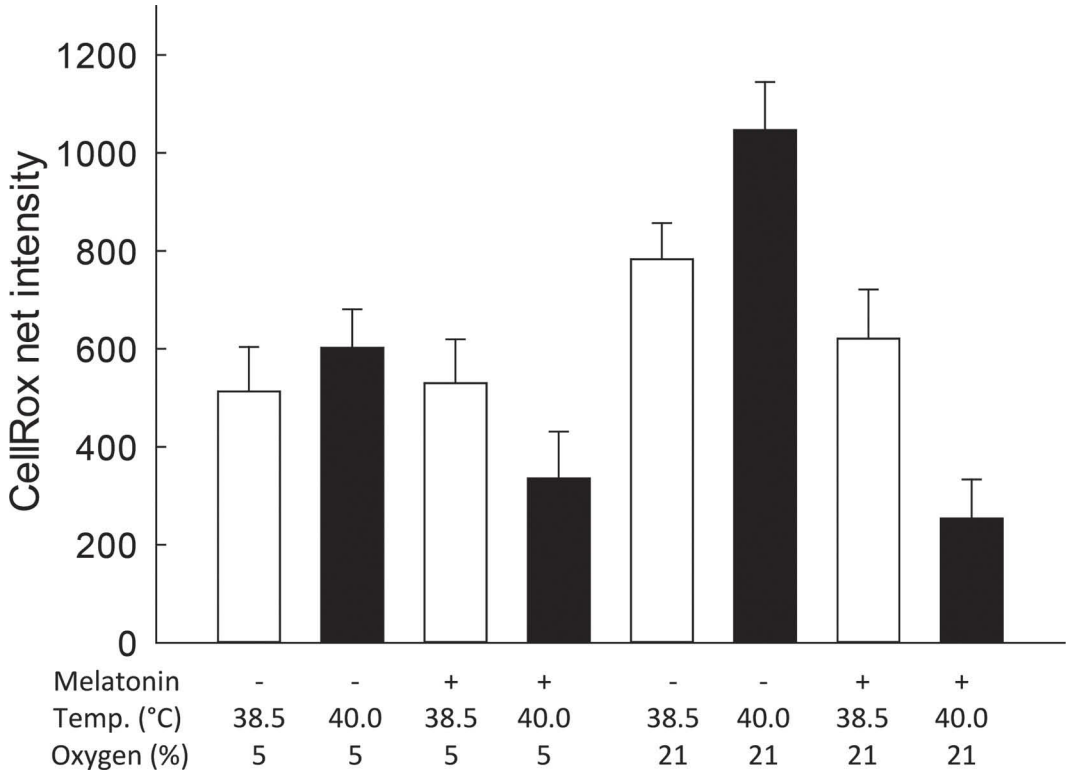

Figure 5. Effect of oxygen environment, incubation temperature (Temp.), and melatonin on production of reactive oxygen species in the zygote as determined by CellROX (ThermoFisher Scientific, Waltham, MA) net intensity (experiment 2). Data are LSM ± SEM of arbitrary intensity units. Intensity was affected by oxygen concentration ($P = 0.004$) and the interaction of temperature with melatonin treatment ($P = 0.0003$) and oxygen with melatonin treatment ($P = 0.0054$).

DISCUSSION

Results of these experiments in which embryos were exposed to heat shock confirm that exposure to elevated temperatures during the zygote stage of development can compromise the competence of the embryo to progress to the blastocyst stage (Sakatani et al., 2012). However, although heat shock resulted in increased ROS production, this action of elevated temperature is not a major cause for the reduced development. This conclusion is based on 2 observations. First, melatonin did not alleviate effects of heat shock on development despite causing a decrease in ROS production. Second, effects of heat shock on development were similar for embryos cultured in a low or high oxygen environment, despite the fact that embryos cultured in high oxygen had higher production of ROS than embryos cultured in low oxygen. The other major conclusion of these experiments is that the genotype for *HSPA1L* affects survival of embryos in a manner that depends upon culture environment. This is because the proportion of blastocysts with a deletion mutation in *HSPA1L* previously related to cellular thermotolerance (Basiricò et al., 2011) was higher for blastocysts derived from heat-shocked zygotes or for embryos cultured at 38.5°C in high oxygen than for those derived from zygotes

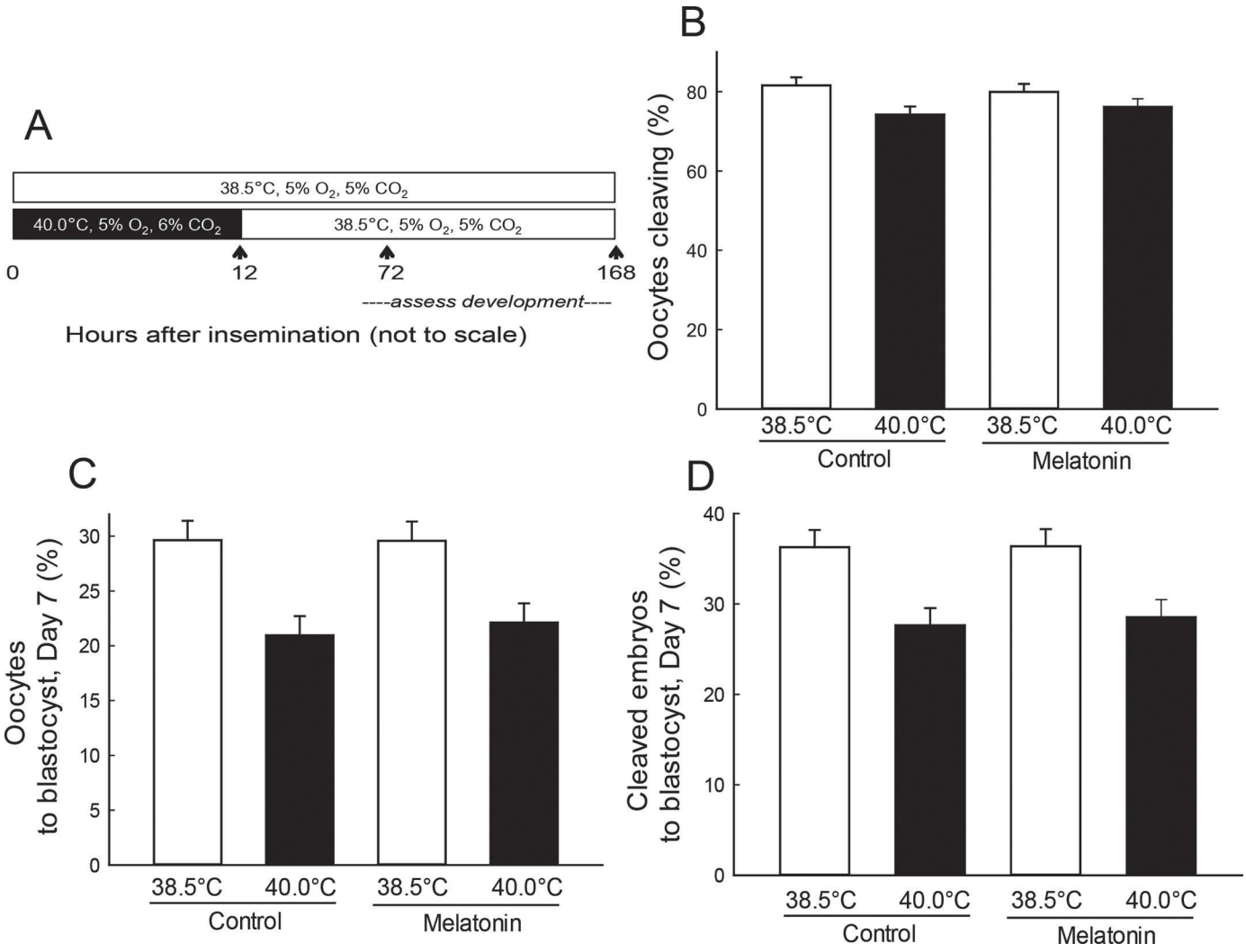

Figure 6. Effect of incubation temperature from 8 to 20 h after fertilization on cleavage and development to the blastocyst stage as affected by melatonin for embryos cultured in low oxygen (experiment 3). Data are LSM ± SEM. The timing and design and timing of treatments is shown in panel A, cleavage rate is shown in panel B, the percent oocytes developing to blastocyst are shown in panel C, and the percent of cleaved embryos developing to blastocyst is shown in panel D. Temperature affected the percent of putative zygotes that cleaved ($P = 0.0012$), the percent that became blastocysts ($P < 0.0001$), and the percent of cleaved embryos that became blastocysts ($P < 0.0001$). No effects were found of melatonin or the interaction between heat shock and melatonin.

not exposed to heat shock and cultured in low oxygen. Thus, there are allelic variants of genes that confer the embryo with protection against adverse environments such as heat shock or high oxygen.

Intracellular ROS are formed by incomplete reduction of oxygen during oxidative phosphorylation and as byproducts of specific enzymatic reactions (Takahashi, 2012). Because of their propensity to strip electrons

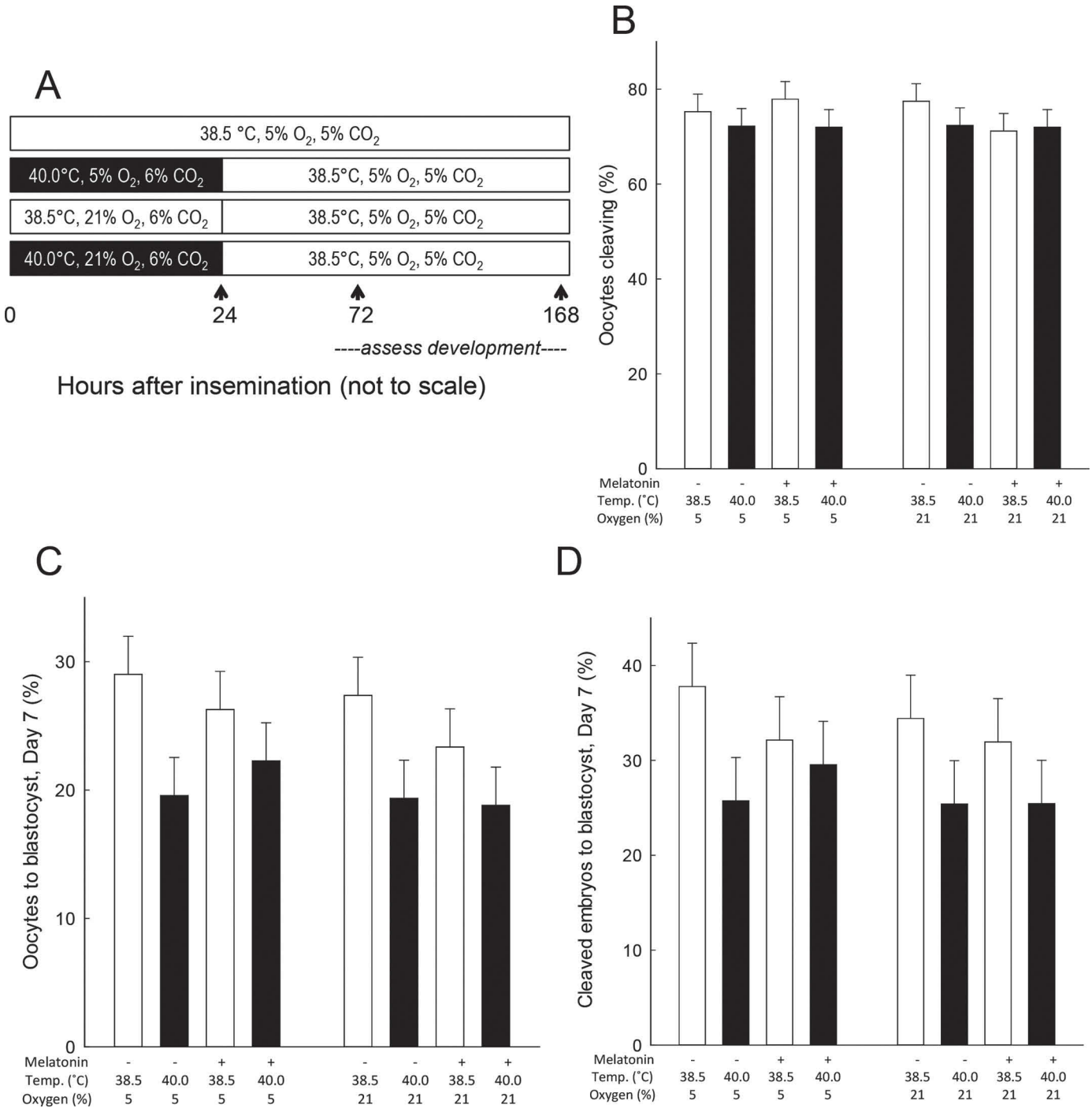

Figure 7. Effect of incubation temperature from 8 to 20 h after fertilization on cleavage and development to the blastocyst stage as affected by oxygen environment during 8 to 20 h after fertilization and melatonin (experiment 4). Data are LSM ± SEM. The timing and design and timing of treatments is shown in panel A, cleavage rate is shown in panel B, the percent oocytes developing to blastocyst is shown in panel C, and the percent of cleaved embryos developing to blastocyst is shown in panel D. Temperature affected the percent of putative zygotes that cleaved ($P = 0.09$), the percent of putative zygotes that became blastocysts ($P = 0.003$), and the percent of cleaved embryos that became blastocysts ($P = 0.01$). Oxygen, melatonin, and interactions with these 2 main effects did not affect any developmental trait.

from other molecules in the cell, ROS can cause cellular damage (Guérin et al., 2001; Ray et al., 2012). As shown here and elsewhere (Sakatani et al., 2004; Nabenishi et al., 2012b), elevated temperature can increase ROS production in embryos. As a result, it has been proposed that increased ROS production is one of the mechanisms by which heat shock reduces embryonic development (Sakatani et al., 2004; Hansen, 2009; Nabenishi et al., 2012a; Hansen, 2013). However, current results are inconsistent with this idea, at least for the bovine zygote. Melatonin did not increase embryonic resistance to heat shock, despite causing a large decrease in ROS production. Likewise, effects of heat shock on development to the blastocyst stage were similar for embryos at high and low oxygen environments, even when ROS production was greater for embryos in a high-oxygen environment. Perhaps the reason why negative effects of high oxygen were not observed in this study is because the exposure to the high oxygen conditions was only for 12 to 24 h and was not enough time to affect embryonic development to the blastocyst stage. Prolonged exposure to high oxygen (24 h or greater) can decrease embryonic development (Papis et al., 2007; Rho et al., 2007; Sakatani et al., 2012). Under prolonged exposure to high oxygen, melatonin can protect the embryo from the damaging consequences of high oxygen (Papis et al., 2007).

In contrast to results with the zygote reported here, melatonin has been reported to protect cultured bovine oocytes exposed to 41.5°C for 24 h in high oxygen maturation from effects of heat shock (Cebrian-Serrano et al., 2013). It is possible that oocytes are more susceptible to ROS than zygotes or that the higher temperatures and incubation periods used increase ROS production beyond what was induced with the lower, but more physiological, heat shock temperatures used here. The effective concentration of melatonin in the experiment of Cebrian-Serrano et al. (2013) was 100 μM versus a concentration of 1 μM in the present experiment. It is unlikely, however, that absence of melatonin effects in the present experiment was because concentrations were too low. Indeed, ROS was greatly reduced by treating heat-shocked zygotes with 1 μM melatonin.

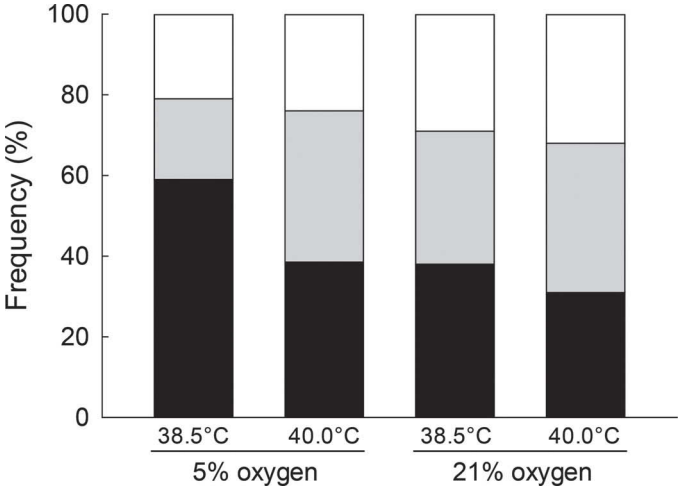

Figure 8. Genotype frequencies of the SNP in *HSPA1L* for blastocysts formed following exposure to different temperatures, oxygen concentrations, and melatonin concentrations (experiment 4). The distribution of blastocysts according to the 3 genotypes [CC (black), CD (gray), and DD (white)] was affected by the following interactions: genotype by temperature ($P < 0.0001$), genotype by oxygen ($P = 0.000184$), and genotype by temperature by oxygen ($P = 0.0054$).

Other antioxidants have been used to reduce effects of heat shock on bovine embryos in vitro and of heat stress in vivo on embryonic survival. Results, which have involved a range of stages of development and culture conditions, have been mixed. No protective effect was found of vitamin E, glutathione, or taurine on preimplantation bovine embryos exposed to heat shock (Ealy et al., 1995; Paula-Lopes et al., 2003a). Similarly, injection of cows with vitamin E (Ealy et al., 1994; Aréchiga et al., 1998b), vitamin E and selenium (Aréchiga et al., 1998a; Paula-Lopes et al., 2003a), or β-carotene (Aréchiga et al., 1998a) did not improve fertility in summer. In contrast, cultured bovine embryos were protected from heat shock by anthocyanin (Sakatani et al., 2007) and dithiothreitol (de Castro e Paula and Hansen, 2008) and fertility of heat-stressed cows was improved by feeding supplemental β-carotene (Aréchiga et al., 1998a).

Although a wide range of responses to altering redox status have been observed, it is likely that the lack of involvement of ROS in the negative effects of heat shock with cultured embryos seen here reflects the situation with respect to heat-stressed cows. That is because, as compared with some other studies, the magnitude of heat shock in terms of duration and temperature reflects body temperatures experienced by heat-stressed cows. Rectal temperatures in lactating dairy cows in the summer in Florida are usually below 40°C (Dikmen and Hansen, 2009; Dikmen et al., 2015). Also, the stage of development studied here, the zygote, is more relevant to the heat-stressed cows because fertility is more likely to be disrupted when cows are exposed to heat stress at d 1 after estrus than later in development (Ealy et al., 1993).

Administration of subcutaneous implants of melatonin have been reported to increase fertility of lactating cows during heat stress (Garcia-Ispierto et al., 2013a). The concentrations of melatonin in the blood in that study ranged from 30 to 65 pg/mL (0.13 to 0.28 nM), which is much lower than tested here (1 μM) and too low to inhibit ROS production (Mayo et al., 2003; Tan

et al., 2003; Rodriguez et al., 2004). Rather it is likely that the effect of melatonin seen by Garcia-Ispierto et al. (2013a) reflects actions of melatonin mediated by specific receptors located in the brain, cumulus cell and oocyte, placenta, and other peripheral tissues (Cardinali et al., 1979; Iwasaki et al., 2005; El-Raey et al., 2011; Slominski et al., 2012). Melatonin has been reported to increase ipsilateral uterine artery blood flow in pregnant Holstein heifers (Brockus et al., 2016), increase circulating progesterone concentration, and decrease serum prolactin in ewes (Kennaway et al., 1982; Abecia et al., 2002), and evidence indicates that melatonin favors cumulus cell expansion in the cow (El-Raey et al., 2011).

We did not determine consequences of heat shock at the 1-cell stage on characteristics of the blastocyst (i.e., cell numbers, incidence of apoptosis, gene expression, and so on). Heat shock during early development can reduce cell number of the resultant blastocysts (Sakatani et al., 2004, 2012). Therefore, it remains possible that, even though melatonin did not block the effects of heat shock on the proportion of embryos that developed to the blastocyst stage, it might have improved the quality of the embryos that did become a blastocyst. Even if this were the case, however, one would not expect that such an action would appreciably improve fertility in heat-stressed cows because most embryos would not reach the blastocyst stage.

The extent to which heat shock negatively affects embryonic development is also controlled in part by the genetics of the embryo. Embryos of thermotolerant breeds are more resistant to the anti-developmental effects of heat shock than embryos of more thermosensitive breeds (Paula-Lopes et al., 2003b; Hernández-Cerón et al., 2004; Eberhardt et al., 2009). Here we show that *HSPA1L* is one gene that contains mutations that confers resistance of embryos to 2 environments: heat shock and high oxygen. The HSP70 family is involved in stabilization of protein structure and inhibition of apoptosis during cellular stress (Radons, 2016). In cattle, 2 genes in the HSP70 family were formed by gene duplication: *HSPA1A* and *HSPA1L* (Grosz et al., 1992). Most studies examining expression of *HSP70* genes have not distinguished between these 2 genes, but a recent study examining the transcriptome of bovine embryos indicates that transcripts for both *HSPA1A* and *HSPA1L* are present in the 2-cell embryo (Jiang et al., 2014). Although embryonic gene activation in cattle occurs for most genes at the 8-cell stage (Graf et al., 2014), transcription of *HSPA1A/L* in response to heat shock occurs as early as the 2-cell stage (Chandolia et al., 1999). The mutation studied here is a deletion in the promoter of *HSPA1L* that, in bovine mononuclear cells, increases upregulation of transcription of the gene in response to elevated temperature and increased viability after heat shock (Basiricò et al., 2011). In Holsteins, the frequency of the deletion mutation has been estimated at 29% (Ortega et al., 2016). In the current study, a similar frequency of the deletion mutation (33%) was observed for blastocysts formed after culture of embryos at 38.5°C in a low oxygen environment. In contrast, frequency of the deletion mutation was increased in blastocysts formed following heat shock under either low or high oxygen conditions or for embryos cultured at 38.5°C in the presence of a high oxygen environment. Such a finding suggests that inheritance of the deletion mutation increased the probability that embryos could develop to the blastocyst stage after exposure to conditions that compromised development (heat shock) or that has been reported to reduce development in other experiments (high oxygen; Papis et al., 2007; Rho et al., 2007; Sakatani et al., 2012). Even though the effect of oxygen on blastocyst development was not significant in experiment 4, the percent of embryos becoming blastocysts was numerically lower in the high oxygen group in the absence of melatonin. A negative effect of high oxygen on development has been observed in multiple experiments (Papis et al., 2007; Rho et al., 2007; Sakatani et al., 2012).

This finding has 2 implications. First, culture of embryos may lead to a skewing of frequency of genes that enhance development under suboptimal culture conditions. In addition, it may be possible to improve embryonic survival during heat stress by selecting for alleles such as the deletion mutation of *HSPA1L* that confer protection from elevated temperature.

CONCLUSIONS

Exposure of bovine zygotes to elevated temperatures reduced competence to develop to the blastocyst stage. This effect is independent of the increased generation of ROS caused by heat shock because reduced development caused by elevated temperature was not alleviated by melatonin or culture in a low oxygen environment even though both treatments reduced generation of ROS. Thus, the previously reported effect of melatonin on fertility of heat-stressed cows might involve actions independent of the antioxidant properties of melatonin. Nevertheless, the effect of melatonin on the oocyte or further embryonic developmental stages should be examined further. Inheritance of a deletion mutation in the promoter region of *HSPA1L* increased the probability that embryos became blastocysts after heat shock or exposure to high oxygen. Thus, genotype may affect whether an embryo survives or not in response

to adverse environments. Perhaps embryonic survival during heat stress could be improved by selecting for thermotolerant genotypes.

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Interpretive summary

Interactions of human chorionic gonadotropin with genotype and parity on fertility responses of lactating dairy cows. *by Adriana Zolini et al.* There is much variability in response of cows to treatments that potentially improve fertility. In this study, it was demonstrated that the effectiveness of treatment of lactating cows with human chorionic gonadotropin (hCG) at day 5 after AI depended on parity and genotype. In particular, hCG was more effective for primiparous cows than multiparous cows, and was associated with a single nucleotide polymorphisms (SNP) in *COQ9*. Fertility of cows treated with vehicle was greatest for the AA allele whereas fertility was lowest for the same genotype for cows treated with hCG. These results indicate that information on a cow's parity and genotype can be used to identify populations of animals that respond positively to fertility treatment.

RUNNING HEAD: GENOTYPE AND PARITY EFFECT ON HCG RESPONSE

Interactions of human chorionic gonadotropin with genotype and parity on fertility responses of lactating dairy cows

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ABSTRACT

Fertility-promoting effects of treatment of lactating dairy cattle with human chorionic gonadotropin (hCG) after artificial insemination (AI) have been variable. Here, we tested whether fertility response to hCG in lactating Holsteins cows interacts with genotype and parity. Primiparous (n=538) and multiparous (n=613) cows were treated with hCG (3,300 IU) or vehicle 5 d after AI. Pregnancy was diagnosed on days 32 and 60 after AI. A subset of cows (n=593 - 701) were genotyped for four single nucleotide polymorphisms (SNP) previously associated with fertility. Treatment with hCG increased progesterone concentration on day 12 after AI regardless of genotype or parity. Pregnancy per AI was improved by hCG in primiparous cows but not in multiparous cows. Moreover, hCG treatment interacted with a SNP in *COQ9* to affect fertility. Fertility of cows treated with vehicle was greatest for the AA allele whereas fertility was lowest for the same genotype among cows treated with hCG. Pregnancy per AI was also affected by genotype for *HSPA1L* and *PGR*, but there were no interactions with treatment. Genotype for a SNP in *PARM1* was not associated with fertility. Overall, results show that variation in response to hCG treatment on fertility depends on parity and interacts with a SNP in *COQ9*.

Keywords: hCG, genotype, parity, SNP, pregnancy, fertility

INTRODUCTION

Personalized medicine is a form of individualized medical therapy in which knowledge from genetics is combined with clinical data to guide clinical treatment. An example of personalized treatment is exclusion of women with breast cancer from tamoxifen treatment based on screening for a SNP in *CYP2D6* associated with reduced function of the enzyme (Schroth et al., 2009; Zeng et al., 2013; Elia et al., 2018). Personalized treatments could conceivably be important in livestock production. Use of genetic information to identify animals for which treatments are likely to be efficacious could reduce the number of animals receiving treatment and thereby achieving cost savings from reduced drug use, labor costs, and withdrawal of milk and meat from the human food chain. Moreover, effectiveness of treatment would increase because only those animals with predisposition for positive response would receive treatment.

In cattle, one type of therapy that could potentially be made more effective through use of personalized medicine is hormonal administration to improve fertility. There is a great deal of between-study variation in effectiveness of some hormonal treatments administered after AI for improving fertility including GnRH (Peters et al., 2000), human chorionic gonadotropin (hCG) (Nascimento et al., 2013), and progesterone (Yan et al., 2016). Some of this variation is likely due to small sample size but there may also be environmental or genetic differences between herds affecting efficacy of treatment.

Here we tested whether effects of one of these hormonal treatments on the fertility of lactating cows depends upon genotype as well as parity. The hormonal therapy tested was hCG treatment at day 5 after AI. Administration of hCG during the early luteal phase can be used to induce ovulation of the first-wave dominate follicle, formation of a functional accessory corpus

luteum and an increase in circulating progesterone concentrations (Schmitt et al., 1996; Santos et al., 2001). Progesterone is critical for pregnancy maintenance and can regulate secretion of proteins and other molecules from the uterine endometrium (Forde et al., 2010) and advance the timing of trophoblast elongation that is a prerequisite inhibition of luteolysis (Lonergan et al., 2013; Lonergan and Forde, 2014). Treatment with hCG at day 5 after artificial insemination (AI) has been reported to improve pregnancy per AI in lactating cows but there is substantial variation in effectiveness of the treatment in the literature (Nascimento et al., 2013). One factor affecting the efficacy of hCG treatment is parity, with hCG treatment being more effective for primiparous females than multiparous animals (Nascimento et al., 2013). Season of the year may also be important because heat stress can decrease circulating concentrations of progesterone (Howell et al., 1994) and treatment with the related molecule, equine chorionic gonadotropin, at day 5 after AI improved pregnancy per AI in lactating cows exposed to heat stress (Garcia-Ispierto et al., 2013).

To evaluate hCG x genotype interactions, animals in the present experiment were genotyped for SNP at 4 loci previously associated with reproductive function in cattle. One SNP is a G → A missense mutation in coenzyme Q9 (*COQ9*) that causes an amino acid change from aspartic acid to asparagine at position 53 of the protein. The A allele is associated with higher genetic merit for daughter pregnancy rate (DPR) and cow conception rate (Cochran et al., 2013b; Ortega et al., 2016a) and higher phenotypic measurements of fertility (Ortega et al., 2017c). The A allele is also associated with more efficient mitochondria in peripheral blood mononuclear cells, and increased mitochondrial DNA copy number in oocytes (Ortega et al., 2017c). Heterozygotes had increased follicle number and antimullerian hormone concentrations than either homozygote (Ortega et al., 2017c). The second SNP is a cytosine deletion in the promoter

region of *HSPA1L* which encodes for a member of the heat shock protein 70 family. This SNP is associated with increased transcript abundance for *HSPA1L* in response to heat shock (Basiricò et al., 2011) and increased survival of cells (Basiricò et al., 2011) and embryos (Ortega et al., 2016b) after heat shock. The mutation is also associated with increased proportion of cultured embryos developing to the blastocyst stage (Cochran et al., 2013b) but with reduced calf crop in Brahman cows (Rosenkrans et al., 2010). The third SNP examined is a G → C missense mutation in prostate androgen-regulated mucin-like protein 1 (*PARM1)* that induces an amino acid change from glycine to alanine at position 232 (Ortega et al., 2017b). The G allele has been associated with higher DPR (Cochran et al., 2013b) and increased embryonic development *in vitro* (Cochran et al., 2013a). The last SNP examined is a C → G mutation in the third intronic region of *PGR* (Driver et al., 2009). The G allele has been related to increased capacity of embryos to develop to the blastocyst stage *in vitro* (Driver et al, 2009), reduced number of oocytes recovered after superovulation (Yang et al., 2011) and increased ability of cows to maintain lower body temperature during the summer (Dikmen et al., 2015).

MATERIALS AND METHODS

Animals and Management

The experiment was conducted on a commercial dairy farm in northern Florida (Alliance Dairy, Trenton, FL, 29°36′54″N 82°49′4″W) with lactating Holstein cows housed in free-stall barns equipped with fans and sprinklers. Cows were milked three times daily and fed a total mixed ration. Animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee. All procedures, including injections, pregnancy diagnosis,

113 blood collection, and timed artificial insemination (TAI) were performed while cows were
114 restrained in self-locking head gates at the feed line.

115 ***Experimental Design and Treatments***

116 Cows were randomly assigned to receive intramuscular injection of either 3,300 IU hCG
117 (Chorulon, Merck Animal Health, Millsboro, DE) or equivalent volume (3.3 ml) of vehicle
118 (diluent) on day 5 after the first insemination following calving. The study was conducted over
119 two years. The first year involved 283 primiparous cows and 489 multiparous cows that received
120 first insemination after calving from June to September 2016, corresponding to hot months of the
121 year. For the second year, the experiment was replicated for 297 primiparous cows receiving first
122 insemination after calving in July and August of 2017.

123 Twice weekly, a group of 20 to 50 cows were enrolled in the Double-Ovsynch protocol
124 (Souza et al., 2008) to allow TAI at 75 to 80 d after calving. Multiparous cows were inseminated
125 with conventional semen and primiparous cows were inseminated with either conventional (n =
126 343) or sexed-sorted (n = 237) semen. At the time of hCG or vehicle injection (day 5 after AI),
127 body condition score (BCS) was estimated as described (Ferguson et al., 1994).

128 For cows in year 1 only, a blood sample (7 mL) was collected on day 12 after AI by
129 puncture of the coccygeal blood vessels using vacutainer tubes containing sodium
130 ethylenediamine tetraacetic acid (Becton & Dickinson, Rutherford, NJ, USA). The samples were
131 placed immediately on ice and brought to the laboratory within 6 h of collection. At the
132 laboratory, 2 mL of blood were collected and stored at – 20°C for later genotype analyses. The
133 remaining sample was centrifuged at 2,000 × g at -4°C for 20 min for separation of plasma.
134 Plasma samples were frozen at – 20°C for later progesterone analyses.

135 Diagnosis of pregnancy was performed by trasnrectal ultrasonography on day 32 ± 3 and
136 60 ± 3 after artificial insemination. A cow was determined pregnant when an embryonic vesicle
137 with a viable embryo (presence of heartbeat) was detected.

138 ***Genotyping***

139 Cows from the first year of the experiment were genotyped for SNP present in *COQ9*
140 (rs109301586), *HSPA1L* (HSP70C895D), *PARM1* (rs111027720), and *PGR* (rs109506766).
141 Details of these SNP are presented in supplemental material in Cochran et al. (2013b). Frozen
142 samples of whole blood were thawed and DNA extraction was performed using the Puregene
143 Blood Core Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions.
144 Genotyping was performed by PCR-based *KASP* assay (LGC Genomics, Middlesex, UK). The
145 assay is a polymerase-chain reaction (PCR)-based technique involving a common reverse primer
146 and two allele-specific forward primers, where one allele-specific primer is labeled with
147 fluorescein amidite and the second to hexachlorofluorescein. Primers used for genotyping are
148 described in Table 1. Depending on SNP, the total number of animals genotyped varied between
149 593 and 701 because of no-calls.

150 Each PCR reaction included 5 μL extracted DNA (20 ng/ml), 5 μL 2× supermix with low
151 ROX (LGC Genomics), and 0.14 μL KASP by design primer mix. Amplification and analysis
152 were performed using a CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA,
153 USA). Thermal cycling conditions were 94°C for 15 min, followed by 10 cycles of 94°C for 20 s
154 and 61°C for 60 s, where the second temperature was decreased 0.6°C per cycle to achieve a
155 final annealing temperature of 55°C at the end of the 10th cycle. The reaction proceeded for an
156 additional 26 cycles of 94°C for 20 s and 55°C for 60 s, and a read step of 37°C for 60 s. To
157 improve genotype cluster visibility, 3 additional cycles of 94°C for 20 s and 55°C for 60 s and a

158 final read step at 37°C for 60 s were performed. In each run, DNA samples from whole blood of
159 cows of known genotypes were analyzed as controls. Determination of the genotype was
160 performed using the allelic discrimination feature of the CFX96 machine.

161 ***Determination of Progesterone Concentrations***

162 Plasma concentrations of progesterone were determined in duplicate using a commercial
163 solid-phase, no-extraction radioimmunoassay (ImmuChem Coated Tube, MP Biomedicals, Costa
164 Mesa, CA). In the five assays performed, quality control samples with concentrations of
165 progesterone representative of the diestrous phase of the estrous cycle (3.7 ng/mL) were
166 included twice (at the beginning and end) of each assay to determine variation within-and across
167 assays. The sensitivity of the assay was 0.1 ng/mL and the average intra-assay and inter-assay
168 CV for the quality control sample was 6.1% and 6.6%.

169 ***Statistical Analysis***

170 Data on pregnancy per AI were analyzed with the GLIMMIX procedure of the Statistical
171 Analysis System (SAS) v 9.4 (SAS Institute, Cary, NC, USA) with the independent variable,
172 pregnancy outcome (pregnant, non-pregnant), modeled as having a binomial distribution.

173 Data from year 1 of the experiment were analyzed using two types of models. The first
174 analysis included effects of hCG treatment, parity, month of insemination, and two-way
175 interactions between hCG treatment, month and parity. BCS was included in the model as a
176 covariate. The second model was used to analyze the effect of each SNP in four separate
177 analyses. For each analysis, the model included hCG treatment, parity, month of insemination,
178 genotype, two-way interactions of hCG treatment with month, parity and genotype and with BCS
179 as a covariate.

An additional analysis of pregnancy data from primiparous cows in years 1 and 2 was performed. The statistical model included effects of hCG treatment, type of semen (sexed vs conventional), month-year of insemination, BCS, and two-way interactions between hCG treatment, month-year and parity.

Effects on progesterone concentrations were first analyzed by GLIMMIX procedure of SAS to estimate the effect of parity and treatmet on the proportion of cows considered to have a functional corpus luteum (progesterone concentration > 1ng/ml) at day 12 after TAI. For this analysis the independent variable, presence of a functional corpus luteum, was modeled as having a binomial distribution. The second set of analyses was peformed by the GLM procedure of SAS and only included data from cows classified as having a functional corpus luteum. The model included hCG treatment, parity, month of insemination, genotype, two-way interactions of hCG treatment with month, parity and genotype and with BCS as a covariate.

For all analyses, a mean separation test using the DIFF option of Proc GLIMMIX was performed to compare differences between individual means for those effects with $P<0.100$ and and that involved multiple degrees-of-freedom. Differences identified by the DIFF option with a $P=0.05$ or less are reported.

RESULTS

Interaction between hCG Treatment and Parity on Pregnancy per AI

In year 1, there was no overall effect of hCG on pregnacy per AI at either d 32 or 60 after insemination. Treatment with hCG tended to increase pregnancy per AI in primiparous cows but not in multiparous cows (Figure 1 A and B). The probability value for the treatment x parity interaction was 0.098 for pregnancy per AI at d 32 and 0.063 for pregnancy per AI at d

60. Inclusion of genotype for *HSPA1L* in the model resulted in the parity x treatment interaction being significant for pregnancy per AI at d 32 of gestation (*P*=0.038) and close to significant at d 60 of gestation (*P*=0.059). Similar results were found for inclusion of genotype for *COQ9* and *PGR* (results not shown).

Analysis of all data from primiparous cows in year 1 and 2 (Figure 1C and D) also indicated that hCG treatment tended to increase pregnancy per AI in primiparous cows. The effect of hCG was *P*=0.159 for pregnancy per AI at d 32 of gestation and *P*=0.063 for pregnancy per AI at d 60 of gestation. There was no effect (P>0.200) of semen type (conventional vs sexed) or interaction with hCG treatment for pregnancy per AI at d 32 or 60.

Interactions of hCG Treatment and Genotype on Pregnancy per AI (Year 1)

The effect of the SNP in *COQ9* and its interaction with hCG treatment is presented in Figure 2. There was a hCG x genotype interaction for pregnancy per AI at d 32 (*P*=0.016) and 60 (*P*=0.012) of gestation. When treated with vehicle, AA cows had a higher pregnancy per AI at d 32 and 60 of gestation than AG cows, with GG cows being intermediate. When treated with hCG, AA cows had lower pregnancy per AI at d 32 than AG cows, with GG cows being intermediate.

Results for the effect of the SNP in *HSPA1L* and its interaction with hCG treatment on pregnancy per AI and response to hCG is presented in Figure 3. Pregnancy per AI was affected by genotype for *HSPA1L* at d 32 (P = 0.009) and 60 (P = 0.001) after TAI. There were, however, no interactions between hCG treatment and *HSPA1L* genotype for pregnancy per AI at either day. For pregnancy per AI at d 32, mean separation tests indicated that CD differed from CC (P=0.005) and DD (P=0.041) but that CC and DD were similar (P>0.10). For pregnancy per AI

at d 60, mean separation tests indicated that CD differed from CC (P=0.001) and DD (P=0.052) and CC and DD were similar (P>0.100).

There was no effect (P>0.100) of the genotype for the SNP in *PARM1* on pregnancy per AI at d 32 or 60 or any interactions between genotype and hCG (Figure 4)**.**

Pregnancy per AI was affected by genotype for *PGR* at d 32 (*P*=0.041) after TAI and tended to be affected (*P* = 0.080) at d 60 after TAI (Figure 5). There was no interaction between the SNP and hCG treatment. For pregnancy per AI at d 32, mean separation tests indicated that CC differed from CG (P=0.034) but other means were not different from each other (P>0.100). For pregnancy per AI at d 60, mean separation tests indicated that CC differed from CG (P=0.025) but other means were not different from each other.

Progesterone Concentrations

Presence of a functional corpus luteum was defined as a progesterone concentration at d 12 after insemination > 1 ng/ml in 90.6% of the primiparous cows and 93.2% of the multiparous cows. Presence of a functional corpus luteum was not affected by parity, hCG treatment or genotype (P>0.100).

Progesterone concentration at d 12 after insemination for animals with a functional corpus luteum was higher (*P*=0.0001) for cows treated with hCG than for cows treated with vehicle (3.2 ± 0.08 vs. 4.6 ± 0.09 ng/mL). The difference in progesterone concentration was greater (interaction, *P*=0.051) for primiparous cows (3.4 ± 0.13 vs. 5.1 ± 0.13 ng/mL) than for multiparous cows (3.1 ± 0.10 vs. 4.3 ± 0.10 ng/mL). Progesterone concentration was not affected by genotype (*P*>0.100) and there were no interactions (*P*>0.100) between genotype and hCG treatment.

DISCUSSION

Here we confirm previous results that fertility response to hCG is greater for primiparous cows than multiparous cows (Nascimento et al., 2013). We also show, for the first time, that there was an interaction between genotype and hCG affecting fertility. In particular, the association between a SNP in *COQ9* previously shown to be related to fertility in cattle (Cochran 2013b, Ortega et al., 2017b) and pregnancy per AI depended upon whether cows were treated with hCG. Associations of SNPs in *HSPA1L* and *PGR* with pregnancy per AI were also found.

The SNP in *COQ9* is a missense mutation in which the A allele has been related to higher fertility in dairy cattle (Cochran et al., 2013b; Ortega et al., 2017b). In the present study, pregnancy per AI among animals treated with vehicle was highest for cows with the AA genotype. Differences among genotypes were altered for cows treated with hCG with AA cows being the least fertile. Indeed, hCG increased pregnancy per AI in AG cows but decreased pregnancy per AI in cows AA cows. Thus, results are indicative that one can use genotyping for *COQ9* to identify animals that would respond positively to hCG as compared to those that will experience, on average, decreased fertility.

The mechanism by which hCG decreased pregnancy per AI in cows with the AA genotype while increasing pregnancy per AI in AG cows is not known. COQ9, along with other COQ proteins (COQ2-COQ8), is involved in the biosynthesis of COQ10 (Tran and Clarke, 2007; Ben-Meir et al., 2015), which is a component of the mitochondrial electron transport system that is required for mitochondrial adenosine triphosphate synthesis. The A allele, which has a frequency of 49-51% in Holsteins (Ortega et al., 2016a), is associated with enhanced mitochondrial function (Ortega et al., 2017c). One possibility is that actions of hCG on reproductive tissues like endometrium (Shemesh et al., 2001) and corpus luteum (Niswender et

al., 2000) have negative consequences when mitochondrial function is enhanced. In the endometrium, activation of LH receptor induces expression of cyclooxygenase which is associated with increased $PGF_{2\alpha}$ production that can trigger luteolysis (Shemesh et al., 2001). Since expression of endometrial LH receptor is low during the luteal phase (Shemesh et al., 2001) treatment with hCG 5 d after TAI may not be able to stimulate enough $PGF_{2\alpha}$ synthesis to trigger luteolysis. However, in cows carrying the AA allele, higher mitochondrial function could conceivably increase cytoplasmic ATP concentration and intensify cellular response to hCG in a way that leads to luteolytic release of $PGF_{2\alpha.}$

There was no effect of *COQ9* genotype on progesterone concentration at d 12 after AI, indicating either no effect of genotype on luteolysis or that luteolysis induced by the interaction between treatment and the SNP for *COQ9* occurs after d 12. Results regarding progesterone response to hCG are also indicative that the relationship between the SNP in *COQ9* and response to hCG was not caused by a differential effect on ability of hCG to induce accessory corpus luteum because progesterone concentrations were similar between groups.

It is also possible that the SNP in *COQ9* is acting through additional mechanisms [note that the SNP is related to follicle number on the ovary; (Ortega et al., 2017c)] or that the SNP is in linkage disequilibrium with a causative mutation located elsewhere in *COQ9* or in other nearby genes.

Although SNP in *HSPA1L* and *PGR* did not affect response to hCG treatment, pregnancy per AI was associated with allelic variation in both genes. For *HSPA1L*, cows that were heterozygous for the locus in *HSPA1L* had a higher pregnancy per AI than cows homozygous for either allele. For *PGR*, CC cows were the least fertile. These results were surprising. There was a lack of association between the mutation in *HSPA1L* and *PGR* for genetic or phenotypic

293 measurements of fertility in Holsteins (Cochran et al., 2013a; Ortega et al., 2016b, 2017a) and
294 the deletion mutation was associated with a reduced proportion of females pregnant during the
295 breeding season for Brahman cows (Rosenkrans et al., 2010). Small sample size could explain
296 some of these discrepanices. In addition, the present experiment was performed during the hot
297 months of the year and it is possible that the increase in pregnacy rate for cows heterozygous for
298 the deletion mutation in *HSPA1L* is related to embryonic resistance to the deleterious effect of
299 heat stress on embryonic survival (Hansen, 2015). The deletion mutation in *HSPA1L,* which
300 encodes for a member of the heat shock protein 70 family, results in increased expression of the
301 protein in response to heat shock (Basiricò et al., 2011) and is associated with cellular resistance
302 to elevated temperature (Basiricò et al., 2011), and increased competence of embryos to develop
303 to the blastocyst stage in culture (Cochran et al., 2013a) and survive to heat shock (Ortega et al.,
304 2016b). Heat stress may also be a factor in the inconsistency in results for *PGR.* The G variant in
305 this gene has been related to maintenance of low body temperature in Holteins cattle during heat
306 stress (Dikmen et al., 2015).

307 Although the G allele in *PARM1* was described to be associated with higher DPR, heifer
308 conception rate and cow conception rate (Cochran et al., 2013b), the mutation in *PARM1* was not
309 associated with pregnacy rate in the present study. Ortega et al. (2016b) also did not find an
310 association between the SNP in *PARM1* and fertility traits. Inconsistency between studies casts
311 doubt on the importance of the SNP in *PARM1* being an important predictor of fertility in
312 Holsteins

313 It was also found that hCG treatment was more effective for primiparous cows than
314 multiparous cows. One possible reason for a higher fertility response to hCG in primiparous
315 cows is that the increase in progesterone concentration at d 12 after insemination caused by hCG

was greater for primiparous cows than multiparous cows. High progesterone concentration during the early pregnancy is associated with higher embryonic elongation and IFNT production (Clemente et al., 2009; Lonergan et al., 2013). In addition, one possible reason for the difference in responses to hCG may reside in the fact that induction of a second ovulation on the side contralateral of the first ovulation reduces pregnancy per AI in multiparous but not primiparous cows (Baez et al., 2017). These authors proposed that the pregnancy response of multiparous cows to formation of a corpus luteum contralateral to the original corpus luteum results in early regression of both structures. Due to the large uterine size in multiparous cows, the physical diffusion of IFNT from the developing conceptus to the contralateral horn may be compromised as well as the maintenance of the accessory CL (Baez et al., 2017).

There are at least two possible explanations for why hCG increased progesterone concentrations to a greater degree for primiparous cows than for multiparous cows. First, it is possible that primiparous cows are more likely to form an accessory CL than multiparous cows. Also, primiparous cows tend to produce less milk than multiparous cows (Nebel and McGilliard, 1993). Progesterone clearance is inversely related to milk yield (Sangsritavong et al., 2002) and differences in rate of metabolism due to parity could affect circulating concentrations of progesterone.

In conclusion, treatment with hCG at d 5 after TAI can increase fertility in primiparous cows exposed to heat stress while not being effective for multiparous cows. In addition, fertility is affected by genotype for *COQ9, HSPA1L* and *PGR* and there is an interaction between hCG treatment and genotype at the *COQ9* locus. This last result is the first evidence in cattle that consideration of genetic information could be informative regarding effectiveness of a fertility treatment. Under current genetic screening systems in cattle, cattle are genotyped at thousands

of individual loci. It is likely that use of all this genetic information can improve the precision of predicting which animals are most likely to respond to therapeutic treatments. Targeting treatment to those animals most likely to respond could increase efficicacy of therapeutics and avoid waste caused by treating animals that will not respond to the therapy.

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479

480 **Table 1.** Nucleotide sequence of the forward and reverse primers used in the PCR-
481 based KASP assay genotype analysis.

Gene	Allele	Primer sequence
COQ9	A	5'-AAGGTCTTTGGATCAGCAGAAGA-3'
	G	5'-AAGGTCTTTGGATCAGCAGAAGG-3'
	Common reverse primer	5'-GAAGAAGAGGGCGGGGGTTGAT-3'
HSPA1L	Deletion	5'-CAAGTCCTGCCCCCTGC-3'
	C	5'-CTCAAGTCCTGCCCCCTGG-3'
	Common reverse primer	5'-GCATCCAGGGCGCTGATTGGTT-3'
PARM1	G	5'-AAGGGCGATGAGGCTGGCG-3'
	C	5'-AAGGGCGATGAGGCTGGCC-3'
	Common reverse primer	5'-TCCCACACTCACCTCCCCTCAA-3'
PGR	G	5'-ACCTAATCTTGAAATAATGGTGATCTAAAG-3'
	C	5'-ACCTAATCTTGAAATAATGGTGATCTAAAC-3'
	Common reverse primer	5'-TCTTATTAATGGTGTCAGCAGATCACCAT-3'

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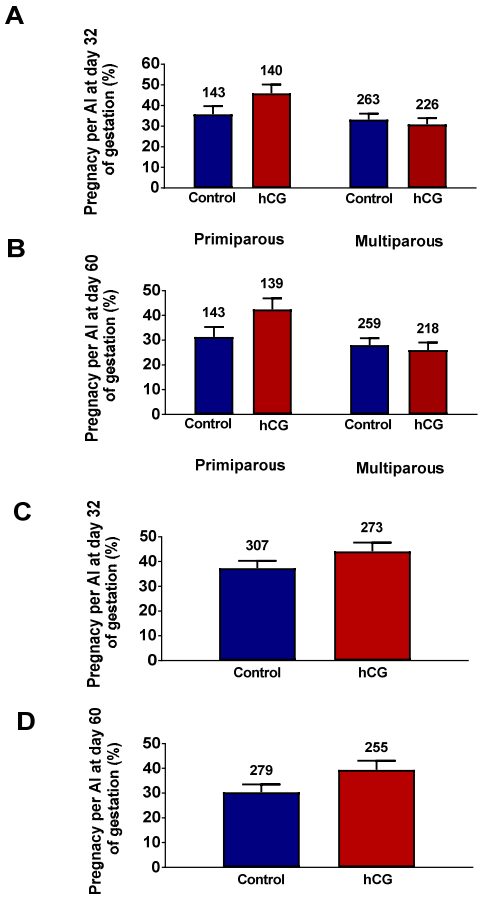
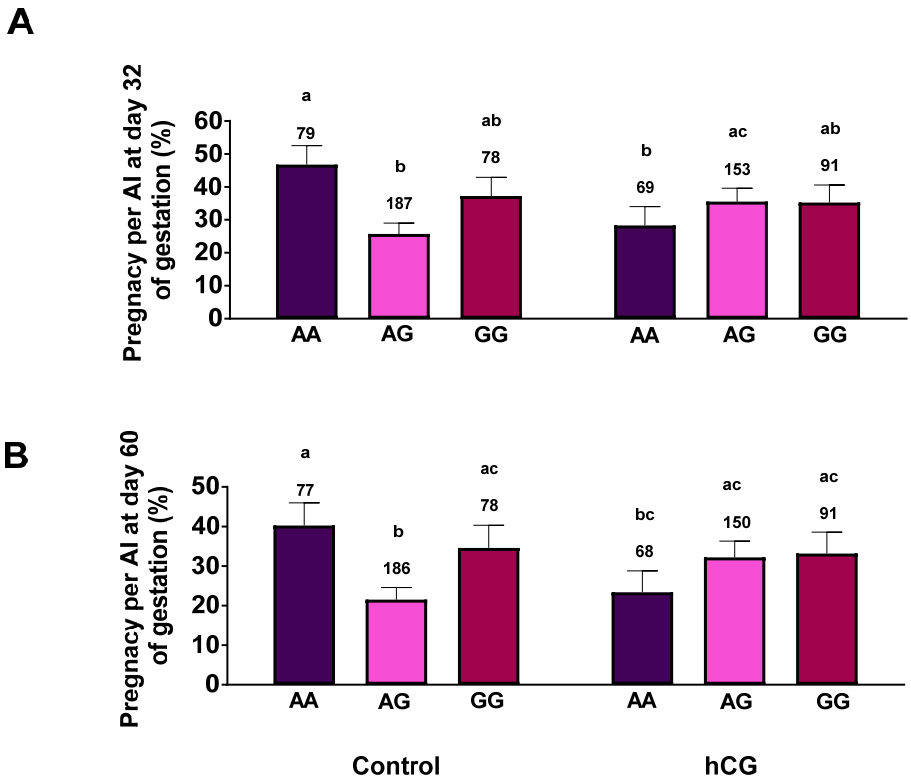


488

489 **Figure 1**. Effect of hCG treatment on pregnancy per AI. Values are least-squares means ± SEM
490 of pregnancy per AI after TAI. Effect of hCG treatment on pregnancy per AI of primiparous **(A)**
491 and multiparous cows **(B)** in year 1. There was a tendency for a treatment x parity interaction on
492 d 32 (A; *P*=0.098) and d 60 (B; *P*=0.063) of gestation, with hCG increasing pregnancy per AI in
493 primiparous cows but not in multiparous cows. **(C and D)** Effect of hCG treatment on pregnancy
494 per AI of primiparous cows (year 1 and 2). The probability value for effect of treatment was
495 *P*=0.159 at d 32 of gestation and *P*=0.063 at d 60 of gestation.

496



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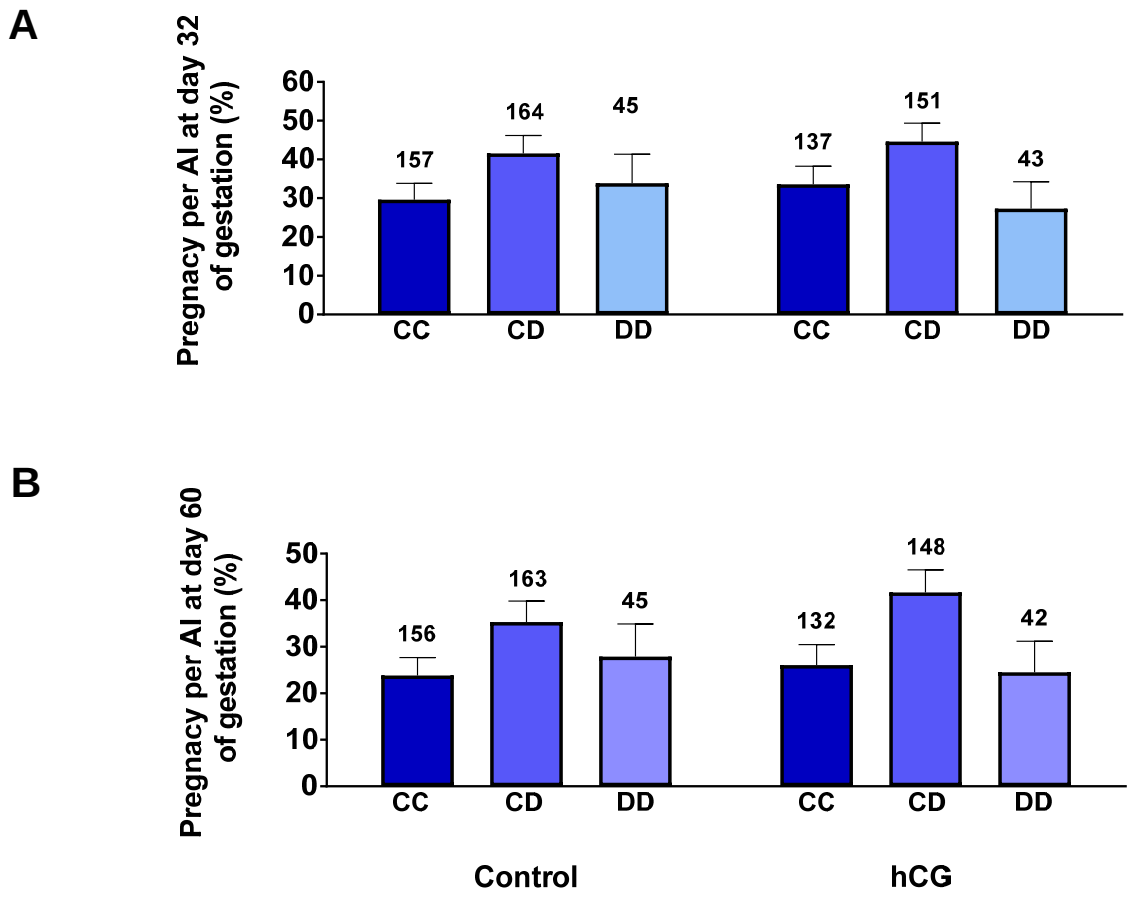
498

499 **Figure 2.** Effect of *COQ9* genotype on the response to hCG treatment on pregnancy per AI.
500 Values are least-squares means ± SEM of pregnancy per AI after TAI. There was a significant
501 interaction between treatment with hCG and genotype for *COQ9* on d 32 [P = 0.016 (**A**)] and d
502 60 [P = 0.012 (**B**)] of gestation. Bars with different superscripts differ (P<0.05). When treated
503 with vehicle, AA cows had a higher pregnancy per AI at d 32 and 60 of gestation than AG cows,
504 with GG cows being intermediate. When treated with hCG, AA cows had lower pregnancy per
505 AI at d 32 than AG cows, with GG cows being intermediate.

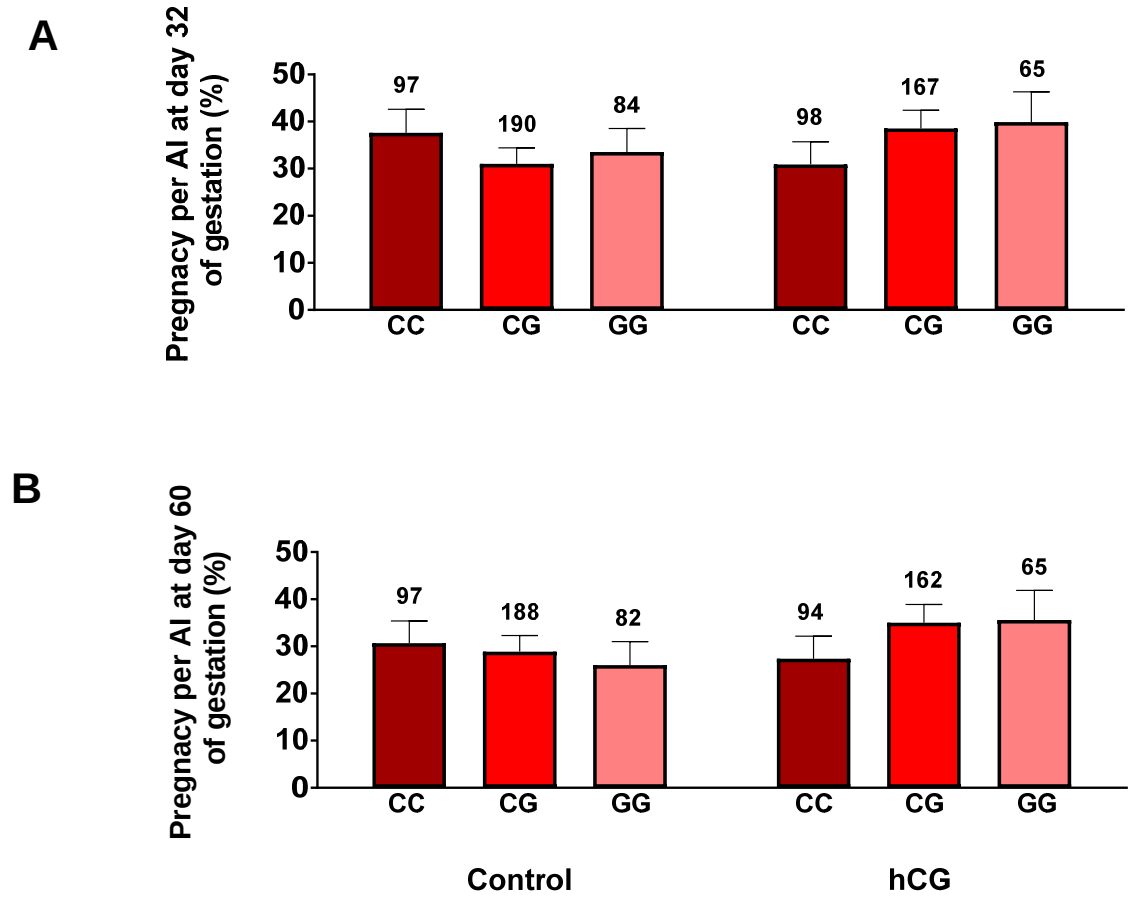
506



507

508 **Figure 3**. Effect of *HSPA1L* genotype on pregnancy per AI. Values are least-squares means ±
509 SEM of pregnancy per AI after TAI. Genotype affected pregnancy per AI at d 32 [*P*=0.009 **(A)**]
510 and 60 [(*P*=0.001 **(B)**] after insemination. For pregnancy per AI at d 32, mean separation tests
511 indicated that CD differed from CC (P=0.005) and DD (P=0.041) but that CC and DD were
512 similar (P>0.10). For pregnancy per AI at d 60, mean separation tests indicated that CD differed
513 from CC (P=0.001) and DD (P=0.052) and CC and DD were similar (P>0.100). There were no
514 interactions between genotype effect and response to hCG treatment.

515



516

517

518 **Figure 4**. Effect of *PARM1* genotype on the response to hCG treatment on pregnancy per AI.

519 Values are least-squares means ± SEM of pregnancy after TAI. There was no effect of *PARM1*

520 on pregnancy per AI and no interaction between treatment with hCG and genotype for *PARM1* at

521 d 32 **(A)** and d 60 **(B)** of gestation.

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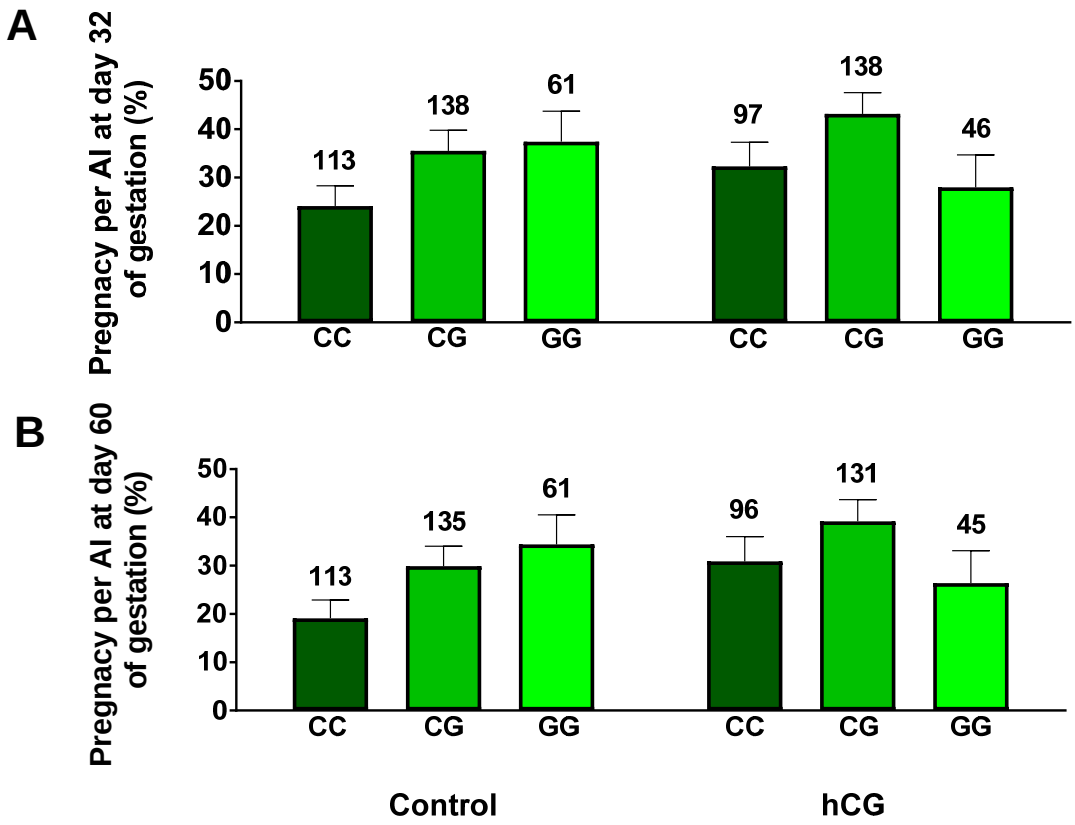

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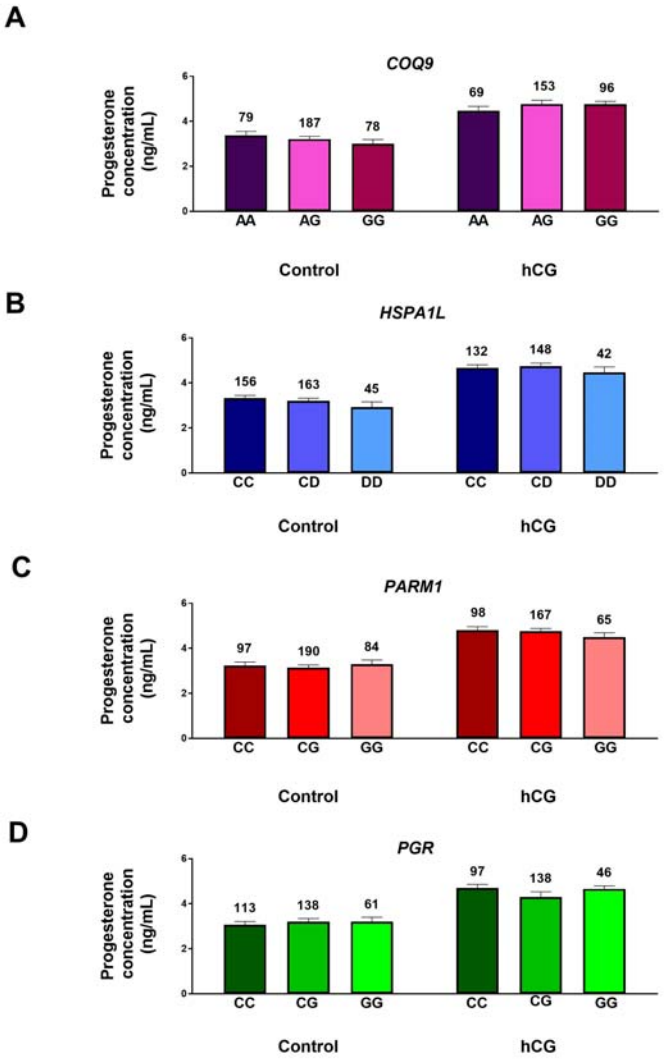
525

526 **Figure 5**. Effect of *PGR* genotype on pregnancy per AI. Values are least-squares means ± SEM
527 of pregnancy per AI after TAI. Genotype affected pregnancy per AI at d 32 [(*P*=0.041) **A**] and
528 tended to affect pregnancy at d 60 [(0.080) **B**] of gestation. For pregnancy per AI at d 32, mean
529 separation tests indicated that CC differed from CG (P=0.012) but other means were not different
530 from each other (P>0.100). For pregnancy per AI at d 60, mean separation tests indicated that
531 CC differed from CG (P=0.025) but other means were not different from each other. There were
532 no interactions between PGR genotype and hCG treatment.



533

534 **Figure 6**. Effects of allelic variation for *COQ9* **(A)**, *HSPA1L* **(B)**, *PARM1* **(C)**, *PGR* **(D)** on
535 progesterone concentration at d 12 after insemination for cows with a functional corpus luteum
536 (progesterone concentrations > 1 ng/mL). Values are least-squares means ± SEM of plasma
537 progesterone concentration. There was no effect of genotype on progesterone concentration and
538 no interaction between treatment with hCG and genotype for any locus on progesterone
539 concentration.

Running title: Melatonin improves oocyte competence

Effects of melatonin on production of reactive oxygen species and developmental competence of bovine oocytes exposed to heat shock and oxidative stress during *in vitro* maturation

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Abstract. Heat shock may disrupt oocyte function by increasing generation of reactive oxygen species (ROS). We evaluated capacity of the antioxidant melatonin to protect oocytes using two models of oxidative stress – heat shock and the prooxidant menadione. Bovine cumulus-oocyte complexes (COC) were exposed in the presence or absence of 1 μM melatonin to the following treatments during maturation: 38.5ºC, 41ºC, and 38.5ºC + 5 μM menadione. In the first experiment, COC were matured for 3 h with 5 μM CellROX® and analyzed by epifluorescence microscopy to quantify production of ROS. The intensity of ROS was greater for oocytes exposed to heat shock and menadione than for control oocytes. Melatonin reduced ROS intensity for heat-shocked oocytes and oocytes exposed to menadione but not for control oocytes. In the second experiment, COC were matured for 22 h. After maturation, oocytes were fertilized and the embryos cultured for 7.5 days. The proportion of oocytes that cleaved after fertilization was lower for oocytes exposed to heat shock and menadione than for control oocytes. Melatonin increased cleavage for heat-shocked oocytes and oocytes exposed to menadione but not for control oocytes. Melatonin tended to increase development for embryos from heat-shocked oocytes but not for embryos from oocytes exposed to menadione or from control oocytes. In conclusion, melatonin reduced production of ROS of maturing oocytes and protected oocytes from deleterious effects of both stresses on competence of the oocyte to cleave after coincubation with sperm. These results suggest that excessive production of ROS compromises oocyte function.

Keywords: Melatonin, heat shock, menadione, reactive oxygen species, embryo.

1. INTRODUCTION

Exposure of cows to thermal stress at estrus can reduce oocyte competence for subsequent development after fertilization (Putney et al., 1988). This action of heat stress is likely to involve direct effects of elevated body temperature on function of the maturing oocyte. Exposure of oocytes during maturation to elevated culture temperature (i.e., heat shock) can disrupt mitochondrial function (Rodriques et al., 2016; Payton et al., 2018) and reduce oocyte competence to complete nuclear maturation (Roth and Hansen, 2005; Nabenishi et al., 2012; Sebrian-Serrano et al., 2013; Meiyu et al., 2015), be fertilized and undergo cleavage (Roth and Hansen, 2004a, 2005, de Castro e Paula and Hansen, 2007; Meiyu et al., 2015). Moreover, competence of the resultant embryos to develop to the blastocyst stage can be compromised (Roth et al., 2004ab; Nabenishi et al., 2012; Rodriques et al., 2016).

Heat shock may disrupt oocyte function, at least in part, by increasing generation of reactive oxygen species (ROS). Production of ROS is increased by heat shock (Nabenishi et al., 2012; Ispada et al., 2018) and antioxidants such as retinol (Lawrence et al., 2004), cysteine (Nabenishi et al., 2012) and astaxanthin (Ispada et al., 2018) can reduce negative consequences of heat shock on the oocyte. Moreover, function of the maturing oocyte can be disrupted by other oxidative stresses, as shown for the nitric oxide donor sodium nitroprusside in the bovine (Soto et al., 2003; Cheuquemán et al., 2015) and hydrogen peroxide in the mouse (Tamura et al., 2008) and pig (Yazaki et al., 2013).

One molecule that may exert protective effects on the oocyte is the multifunctional hormone melatonin (N-acetyl-5-methoxytryptamine). Administration of melatonin to heat-

stressed females improved embryo competence for development in mice (Matsuzuka et al., 2005) and fertility in lactating cows (Garcia-Ispierto et al., 2013). Melatonin affects cellular function by interacting with membrane and nuclear receptors and by functioning as an antioxidant (Tan et al., 2002; Mayo et al., 2017). Melatonin improved oocyte maturation in the pig (Shi et al., 2009), sheep (Tian et al., 2017) and bovine (El-Raey et al., 2011; Tian et al., 2014; Marques et al., 2018).

Experiments to test whether melatonin protects oocytes from heat shock have yielded inconclusive results, however. Effects of melatonin in the pig were evaluated in heat-shocked oocytes but not in oocytes cultured at normal temperature (Li et al., 2015, 2016). Protective effects of high concentrations of melatonin (10 mM) on bovine oocytes cultured at 41.5°C were difficult to interpret because 10 mM melatonin reduced oocyte competence in the absence of heat shock (Cebrian-Serrano et al., 2013).

In the current experiment, we evaluated capacity of melatonin to protect oocytes using two models of oxidative stress. In addition to heat shock, cytoprotective properties of melatonin against the prooxidant menadione were examined. Menadione, (2-methyl-l,4-naphtoquinone), is a vitamin K precursor that can react with a single electron to produce a free radical derivative that can cause superoxide anion formation (Comporti, 1989). Menadione can reduce developmental capacity of the preimplantation bovine embryo (Moss et al., 2009) and act on bovine spermatozoa to reduce fertilizing ability and compromise developmental competence of the resultant embryos (Hendricks and Hansen, 2010).

2. MATERIALS AND METHODS

2.1 Oocyte collection and maturation

Ovaries were obtained from a local abattoir from cows of a variety of genotypes including *Bos taurus* and admixtures of *B. taurus* and *B. indicus*. Ovaries were transported within 10 h to the laboratory at 23°C in a solution of 0.9% (w/v) NaCl. Cumulus-oocyte complexes (COC) were harvested from follicles 2 to 8 mm in diameter by cutting the surface of the ovary with a scalpel and swirling the ovary in BoviPRO^{TM} Oocyte Washing Medium (with BSA) (MOFA Global, Verona, WI, USA). Only COC with at least 3 to 4 layers of compact cumulus and with an oocyte with a uniform cytoplasm were selected for maturation. Selected COC were washed and matured in 6-well plates in groups of 25-30 in 300 µL BO-IVM oocyte maturation medium (IVF Bioscience, Falmouth, UK) that was prepared $\pm$ 1 µM melatonin (Santa Cruz Biotechnology, Dallas, TX, USA) and with either 5 µM menadione (Sigma-Aldrich, St. Louis, MO, USA) or an equivalent volume of vehicle. Melatonin was prepared as described by Ortega et al. (2016) and menadione as described by Moss et al. (2009). Depending on the experiment, maturation was carried out for 3 or 22 h at 38.5ºC or 41.0ºC under an atmosphere of either 5% (v/v) CO_2 in humidified air (38.5°C) or 7% (v/v) CO_2 in humidified air (41.0°C). The higher CO_2 for maturation at 41.0°C was used to maintain pH at 7.4 in the face of lower solubility of CO_2 at the higher temperature.

2.2 Production of ROS

An experiment was conducted to determine whether heat shock and menadione would increase production of ROS by oocytes at the beginning of exposure to stress and whether increased production would be blocked by melatonin. For each replicate, COC (~100) were

104 randomly assigned to one of 6 treatments in a 3 x 2 factorial arrangement with main effects
105 of stress (control, heat shock, or menadione) and melatonin (0 or 1 μM). Controls were
106 cultured at 38.5°C, heat shock was 41.0°C and menadione involved culture at 38.5°C in
107 BO-IVM containing 5 μM menadione. For all treatments, medium contained 5 μM
108 CellROX® Green, a cell-permeant dye that exhibits bright green photostable fluorescence
109 upon oxidation and subsequent binding to DNA.

110 After 3 h of maturation, COCs were denuded of cumulus cells by vortexing groups
111 of 25 to 30 for 5 min in 200 μL hyaluronidase as described earlier (Ortega et al., 2017).
112 Oocytes were then washed 3 times in 50-μL droplets of Dulbecco's phosphate-buffered
113 saline (DPBS) containing 1% (w/v) polyvinylpyrrolidone (PVP), fixed in 4% (w/v)
114 paraformaldehyde in DPBS, washed 3 more times in DPBS-PVP and mounted in groups of
115 10 oocytes on microscope slides using Prolong Gold anti-fade reagent (Invitrogen,
116 Carlsbad, CA, USA). Oocytes were examined for fluorescence within 10 h after labeling
117 by fluorescence microscopy using a green emission filter with a Zeiss Axioplan 2
118 epifluorescence microscope (Zeiss, Göttingen, Germany). Digital images of each oocyte
119 were acquired using AxioVision software (v. 4.8.2, Zeiss) and a high-resolution black and
120 white Zeiss Axiocam MRM digital camera. Analysis of the images was performed using
121 ImageJ V. 1.48 (National Institutes of Health, Bethesda, MD, USA). Net fluorescent
122 intensity was calculated by obtaining the average pixel intensity of each oocyte (obtained
123 after manually drawing a boundary around the oocyte) and subtracting the background
124 intensity obtained from a region of the image not containing the oocyte.

The experiment was replicated four times using a total of 41-72 oocytes per treatment (n=326 total).

2.3 Competence of oocytes to cleave and develop after fertilization

An experiment was conducted to determine whether 1) heat shock and menadione would alter competence of matured oocytes to cleave after fertilization and alter ability of the resultant embryos to develop to the blastocyst stage and 2) if actions of heat shock and menadione would be blocked by melatonin. For each replicate (n=12 replicates total), COC (~200) were randomly assigned to one of 6 treatments in a 3 x 2 factorial arrangement with main effects of stress (control, heat shock, or menadione) and melatonin (0 or 1 μM). Control COC were matured for 22 h at 38.5°C, heat shocked COC were matured for 14 h at 41.0°C and for 8 h at 38.5°C, and menadione-treated COC were matured for 22 h at 38.5°C in medium containing 5 μM menadione.

After maturation, COC were washed 3 times in HEPES-TALP (Tyrodes albumin-lactate-pyruvate) and placed in wells of 6-well plates containing 425 μL fertilization medium [in vitro fertilization-TALP; see Ortega et al. (2017) for recipes for TALP media] and 1×10^6/mL spermatozoa. For each replicate, fertilization was performed with semen pooled from 3 individual bulls of various taurine breeds; the total number of bulls used in the experiment was 17. Sperm were purified from frozen-thawed straws of extended semen using an Isolate® gradient [Irvine Scientific, Santa Ana, CA USA; 50% (v/v) and 90% (v/v) Isolate]. In addition, 20 μL of penicillamine-hypotaurine-epinephrine solution (Ortega et al., 2017) was added to each fertilization well to improve sperm motility.

Fertilization proceeded for 14 to 16 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air.

Putative zygotes (i.e., oocytes exposed to sperm) were denuded from the surrounding cumulus cells at the end of fertilization (14 to 16 h) by vortexing groups of 25 to 30 zygotes for 5 min in 200 µL of HEPES-TALP containing 10,000 U/mL of hyaluronidase. Embryos were cultured in groups of 25-30 in 50 µL drops of culture medium (SOF-BE2; Ortega et al., 2017) that were covered with mineral oil. Embryos were cultured at 38.5°C in a humidified atmosphere of 5% (vol/vol) O_2 and 5% (vol/vol) CO_2 with the balance N_2. The percent of oocytes that cleaved was determined at Day 3.5 of development (Day 0 = day of fertilization) and the percent of cleaved embryos that became blastocysts was determined at Day 7.5 of development.

The experiment was replicated 12 times with the total number of COC ranging from 299 to 444 per treatment (total number = 2491).

2.4 Statistical analysis

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). Data on ROS production were analyzed by analysis of variance using the MIXED procedure. The model included main effects of stress and melatonin and the interaction of stress and melatonin as fixed effects and replicate as a random effect. Effects of stress and melatonin on the proportion of oocytes cleaving and on the proportion of cleaved embryos becoming blastocysts was evaluated using the GLIMMIX procedure. Each embryo was considered an observation with binary response (0 = not developed to blastocyst, 1 = developed to blastocyst) and analysis was performed by logistic regression fitting binary data

distribution. The statistical model included the fixed effects of stress, melatonin, stress x melatonin interaction and random effect of replicate.

For both analyses, two sets of orthogonal contrasts were used to make individual degree-of-freedom comparisons of resolve multilevel effects of stress and interactions of stress and melatonin. In the first set of contrasts, differences between types of stress (control, heat shock, and menadione) were evaluated by two orthogonal contrasts: 1) the comparison of control vs heat shock + menadione and 2) the comparison of heat shock vs. menadione. In the second set of contrasts, differences in the effect of melatonin for each stress were evaluated by comparisons of 1) control vs control + melatonin, 2) heat shock vs heat shock + melatonin and 3) menadione vs menadione + melatonin.

3. RESULTS

3.1 Production of ROS

Representative images of labeling of oocytes using CellROX are shown in Figure 1. Intensity of fluorescence was higher for oocytes exposed to heat shock (41.0°C) or 5 μM menadione than for oocytes matured at 38.5˚C (compare Figure 1C and Figure 1E with Figure 1A). Addition of 1 μM melatonin reduced fluorescent intensity under all culture conditions (compare Figure 1A, Figure 1C and Figure 1E with Figure 1B, Figure 1D and Figure 1F).

Results of quantification of ROS labeling are shown in Figure 2. The intensity of ROS was greater (P=0.0577) for oocytes exposed to heat shock and menadione than for control oocytes. Overall, melatonin reduced ROS intensity (P=0.0002) and there was no

interaction between stress and melatonin (P=0.4806). However, analysis of the effects of melatonin for each stress indicated that melatonin reduced ROS intensity for heat-shocked oocytes (P=0.0305) and oocytes exposed to menadione (P=0.0007) but not for control oocytes (P=0.2002).

3.2 Competence of oocytes to cleave and develop after fertilization

Results on cleavage of oocytes after fertilization are presented in Figure 3A. The proportion of oocytes that cleaved after fertilization was lower (P<0.0001) for oocytes exposed to heat shock and menadione than for control oocytes. Overall, melatonin increased cleavage (P=0.0041). While the interaction between stress and melatonin was not significant (P=0.2944), analysis of the effects of melatonin for each stress indicated that melatonin increased cleavage for heat-shocked oocytes (P=0.0305) and oocytes exposed to menadione (P=0.0122) but not for control oocytes (P=0.6675).

As shown in Figure 3B, the proportion of cleaved embryos that became blastocysts was not affected by stress (heat shock + menadione vs control, P=0.7871) but was increased by melatonin (P=0.0634). Despite a lack of a stress x melatonin interaction (P=0.5991), analysis of effects of melatonin for each stress indicated that melatonin tended to increase development for embryos from heat-shocked oocytes (P=0.0702) but not for embryos from oocytes exposed to menadione (P=0.5426) or from control oocytes (P=0.4384).

4. DISCUSSION

Present results indicate that melatonin can reduce ROS production by the bovine oocyte exposed to conditions that promote production of ROS and partially preserve

developmental competence of the oocyte exposed to those stresses. These results confirm the importance of oxidative stress for damaging the oocyte and show how administration of an antioxidant can block that effect.

It has been repeatedly demonstrated, both in the present experiments and by others, that heat shock increases ROS production by the bovine oocyte (Nabenishi et al., 2012; Ispada et al., 2018) and reduces the percent of oocytes that cleave after coincubation with spermatozoa (Roth and Hansen, 2004a; de Castro e Paula and Hansen, 2007). In some cases, the deleterious actions of heat shock on the oocyte also compromise the ability of the subsequent embryo to develop to the blastocyst stage (Roth et al., 2004ab; Nabenishi et al., 2012; Rodriques et al., 2016) although this consequence of heat shock has not always observed (de Castro e Paula and Hansen, 2007; Sebrian-Serrano et al., 2013). In the present experiment, the percent of cleaved embryos becoming blastocysts for oocytes cultured without melatonin was 26.7% for control oocytes and 22.0% for heat-shocked oocytes. Thus, the primary defect caused by heat shock here was the competence of the oocyte to cleave after fertilization. Treatment of the oocyte with the prooxidant menadione also reduced percent of oocytes that cleaved while not affecting subsequent development of cleaved embryos. Previous experiments with heat shock would indicate that reduced competence for cleavage is due to disruption of nuclear maturation (Roth and Hansen, 2005; Nabenishi et al., 2012; Sebrian-Serrano et al., 2013; Meiyu et al., 2915) as well as disruption of mitochondrial function (Rodrigues et al., 2016; Payton et al., 2018) and induction of oocyte apoptosis (Roth and Hansen, 2004a, 2005; Meiyu et al., 2015).

Deleterious effects of both heat shock and menadione on percent of oocytes that cleaved were reduced by melatonin. Moreover, there was a tendency for melatonin to increase the competence of embryos derived from heat-shocked oocytes to develop to the blastocyst stage. Earlier experiments to evaluate the thermoprotective effect of melatonin on the oocyte have been difficult to interpret either because of lack of control oocytes not exposed to heat shock (Li et al., 2015, 2016) or the high concentrations of melatonin (10 mM) required to protect oocytes from heat shock reduced oocyte competence in the absence of heat shock (Cebrian-Serrano et al., 2013). The experiments conducted here did not allow determination of whether the cytoprotective effects of melatonin were mediated by reducing ROS production or through changes in cellular function mediated by activation of melatonin receptors. The former explanation seems more likely because addition of melatonin caused a large reduction in ROS generation, melatonin was protective against two different stresses (heat shock and menadione) that both increase ROS production, and there were little effects of melatonin on oocyte competence in the absence of heat shock or menadione. There are reports of the existence of melatonin receptors or their mRNA in the bovine oocyte and cumulus cell (El-Raey et al., 2011; Tian et al., 2014) and further studies are necessary to resolve the mechanism of action of the cytoprotective effects of melatonin on the oocyte.

An additional indication of the mechanism of action of melatonin is the effective concentration of the hormone. The concentration of melatonin used here, 1 μM, is much higher than the reported Kd of membrane melatonin receptors, having values of ~30-225 pM (Poon et al., 1994; Kobayashi et al., 2003; Liu et al., 2013). In an earlier study, there

255 was no thermoprotective effect of 1 pM or 1 nM melatonin on bovine oocytes exposed to
256 heat shock (Cebrian-Serrano et al., 2013).

257 There is a report that administration of melatonin to cows via subcutaneous
258 implants can improve fertility of heat-stressed cows (Garcia-Ispierto et al., 2013). In that
259 study, concentrations of melatonin in peripheral blood of treated cows peaked at 60-70
260 pg/mL (i..e, 260-300 pM). Thus, beneficial effects of melatonin in that study may have
261 involved receptor-mediated actions of melatonin on one or more components of the
262 reproductive system rather than a direct cytoprotective action of the molecule.

263 The finding that 1 μM melatonin reduced effects of heat shock and melatonin on
264 the oocyte is in contrast with recent results with the bovine two-cell embryo. Culture of
265 embryos at this stage of development causes an increase in ROS production and a decrease
266 in the proportion of embryos that develop to the blastocyst stage (Ortega et al., 2016).
267 While melatonin treatment blocked the increase in ROS production caused by heat shock,
268 it did not rescue embryos in terms of development to the blastocyst stage. One explanation
269 for this difference between the oocyte and embryo is that ROS are a more important
270 mediator of heat shock effects in the oocyte than in the two-cell embryo.

271 In conclusion, melatonin reduced production of ROS by maturing oocytes,
272 especially when they were exposed to heat shock or menadione, and protected oocytes
273 from deleterious effects of both stresses on competence to cleave after coincubation with
274 sperm. These results suggest that excessive production of ROS compromises oocyte
275 function. Such a mechanism may be important for effects of elevated temperature on
276 damage to the oocyte during maturation.

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285 **Conflict of interest**

286 The authors have no conflict to declare.

287

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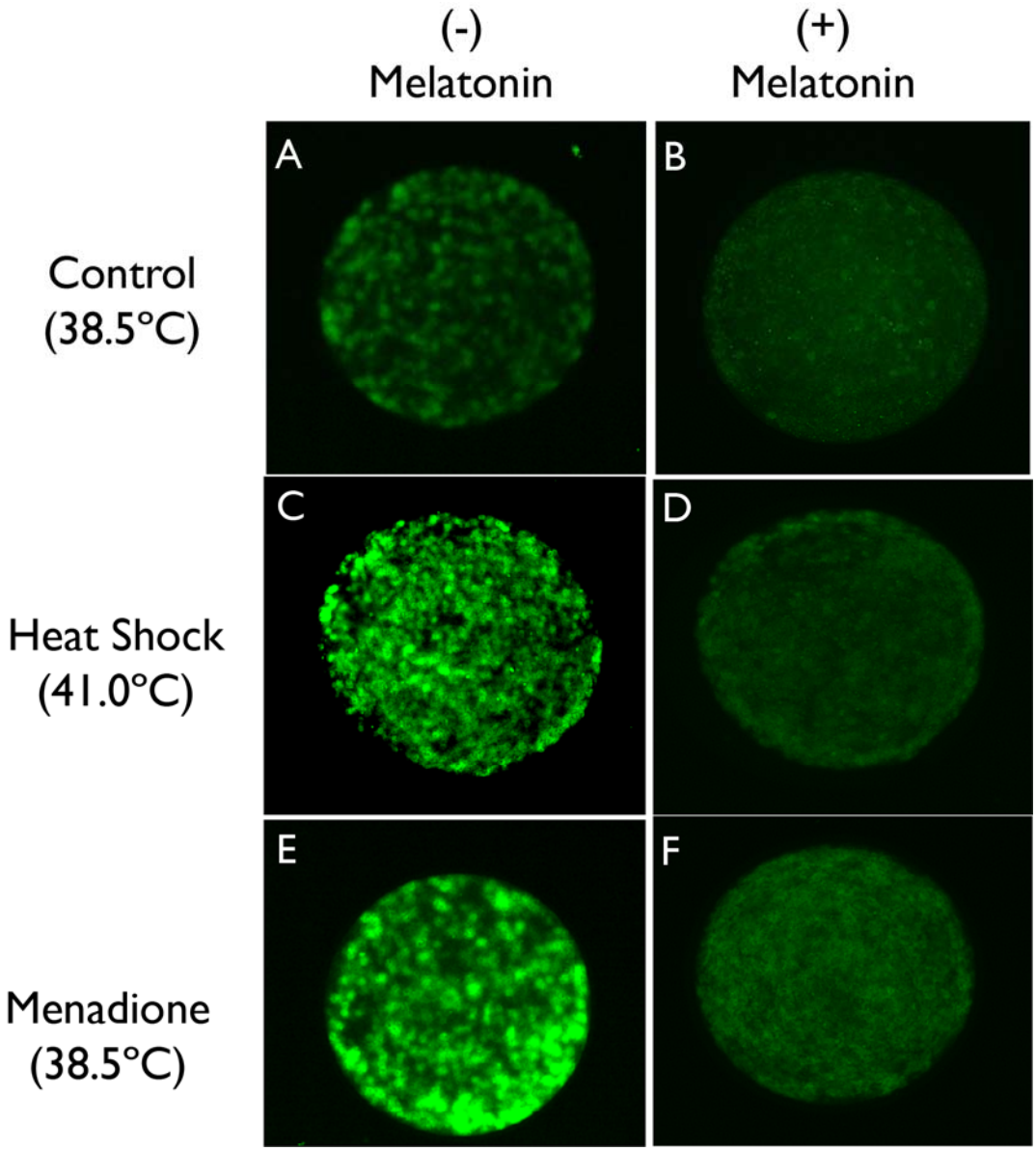

398

399 **FIGURE. 1.** Representative images of oocytes labeled with CellROX (ThermoFisher
400 Scientific, Waltham, MA) to assess production of ROS as affected by incubation
401 temperature, menadione and melatonin.

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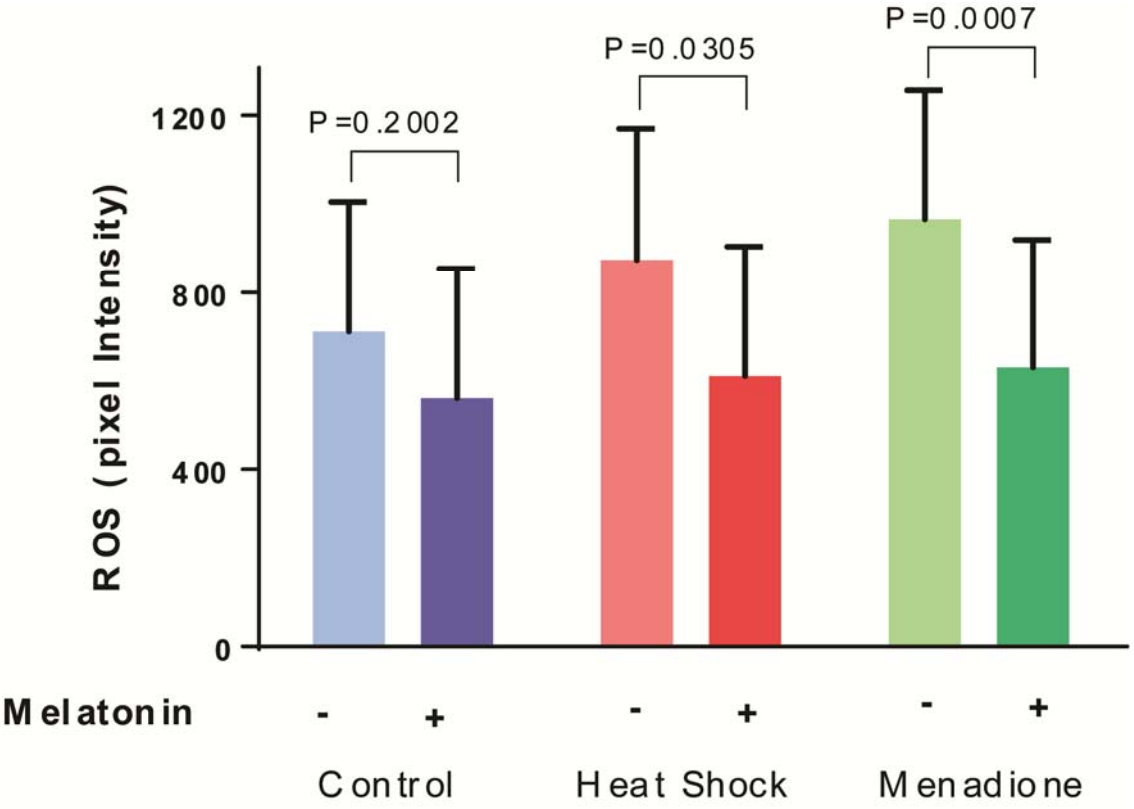
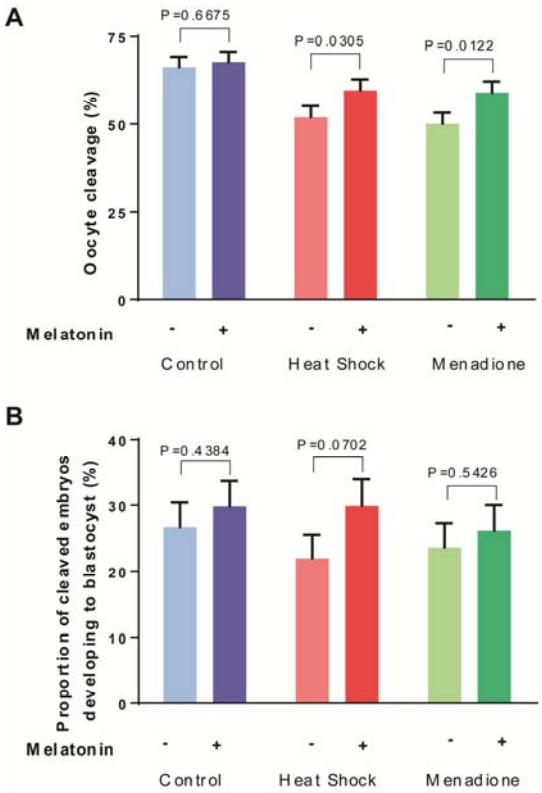


403

404 **FIGURE 2.** Effects of melatonin (1 μM) on production of reactive oxygen species (ROS)
405 by maturing oocytes exposed to control conditions (38.5ºC), heat shock (41.0ºC) and
406 menadione (5 μM at 38.5ºC). Data are least-squares means ± SEM of pixel intensity.
407 Overall, the intensity of ROS was greater (P=0.0577) for oocytes exposed to heat shock
408 and menadione than for control oocytes. Probability values for the effect of melatonin for
409 each stress are indicated above the bars.

410



411

412 **FIGURE 3.** Effects of melatonin (1 µM) on developmental competence of oocytes
413 exposed to control conditions (38.5ºC), heat shock (41.0ºC) and menadione (5 µM at
414 38.5ºC) during *in vitro* maturation. Data are least-squares means ± SEM of proportion of
415 oocytes that cleaved after fertilization (A) and the proportion of cleaved embryos
416 developing to the blastocyst stage (B). Overall, the proportion of oocytes that cleaved after
417 fertilization was lower (P<0.0001) for oocytes exposed to heat shock and menadione than
418 for control oocytes. The proportion of cleaved embryos that became blastocysts was not
419 affected by stress (heat shock + menadione vs control, P=0.7871). Probability values for
420 the effect of melatonin for each stress are indicated above the bars.

Follicular fluid exosomes act on the bovine oocyte to improve oocyte competence to support development and survive heat shock

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Running head: Exosomes enhance oocyte function

Summary sentence: Bovine follicular fluid exosomes increase oocyte competence to support development and survive heat shock.

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Abstract. Addition of follicular fluid to oocyte maturation medium can affect cumulus cell function, increase competence of the oocytes to be fertilized and develop to the blastocyst stage, and protect the oocyte from heat shock. Here, it was tested whether exosomes in follicular fluid are responsible for effects of follicular fluid on function of the cumulus-oocyte complex (COC). This was accomplished by culturing COC during oocyte maturation at 38.5°C (body temperature of the cow) or 41°C (heat shock) with follicular fluid or exosomes derived from follicular fluid and evaluating various aspects of function of the oocyte and embryo derived from it. Both follicular fluid and exosomes increased cumulus cell expansion and the percent of oocytes that cleaved and developed to the blastocyst stage. Negative effects of heat shock on cleavage and blastocyst development, but not cumulus expansion, were reduced by follicular fluid and exosomes. Fluorescently labelled exosomes were taken up by cumulus cells but not the oocyte, suggesting that actions of follicular fluid exosomes are mediated through changes in cumulus cell function. Results support the idea that exosomes in follicular fluid play important roles during oocyte maturation to enhance oocyte function and protect it from stress.

Additional keywords: Exosomes, oocyte maturation, heat shock

Introduction

The thermal environment is an important determinant of fertility in mammals with heat stress in particular compromising sexual behavior, gametogenesis and fertility (Hansen, 2009). In the bovine, for example, the percent of lactating cows in Israel that became pregnant averaged 39-40% for cows bred in winter vs 3-25% for cows bred in summer (Flamenbaum and Galon, 2010). One cause of reduced fertility in heat-stressed females is damage to the oocyte. Heat stress can reduce oocyte function as early as 100 days before ovulation (Torres-Júnior et al., 2008) and as late as the peri-ovulatory period when the oocyte undergoes nuclear and cytoplasmic maturation (Putney et al., 1988).

Reduced oocyte function during heat stress is caused, at least in part, by damage to the oocyte caused by exposure to elevated body temperatures (i.e., heat shock). Exposure of oocytes to elevated temperatures during maturation leads to cytoskeletal alterations (Roth and Hansen, 2005), induction of apoptosis (Roth and Hansen, 2004a, 2005; Rodrigues et al., 2016), mitochondrial dysfunction (Rodrigues et al., 2016), increased generation of reactive oxygen species (Nabenishi et al., 2012; Ispada et al., 2018), compromised nuclear maturation (Roth and Hansen, 2005; Nabenishi et al., 2012), reduction in fertilization (Roth and Hansen, 2005) and cleavage (Roth and Hansen, 2004a; de Castro e Paula and Hansen, 2007). Additionally, cleaved embryos derived from heat-shocked oocytes had reduced capacity to develop to the blastocyst stage (Roth and Hansen, 2004b; Nabenishi et al., 2012; Rodrigues et al., 2016).

Oocyte maturation takes place in the follicle (Mehlmann, 2005) and it is possible that regulatory molecules produced by cumulus cells adjacent to the oocyte or present in follicular fluid could protect the oocyte from heat shock. Indeed, addition of either follicular fluid or insulin-like growth factor 1 (IGF1) to maturation medium reduced effects of elevated culture

temperature on oocyte cleavage and development of the resultant embryo to the blastocyst stage (Rodrigues et al., 2015, 2016). One component of follicular fluid that could potentially be involved in regulation of oocyte function in response to heat shock is the pool of exosomes. These extracellular vesicles interact with target cells through either internalization and delivery of cargo molecules to the cytoplasm or endosome or via interaction with membrane receptors on the cell surface without internalization (Urbanelli et al., 2013). In the cow, follicular exosomes have been shown to alter transcript abundance in oocytes (Dalenezi and Ferreira, 2016) and granulosa cells (Sohel et al., 2013; Hung et al., 2015), increase cumulus expansion during oocyte maturation (Hung et al., 2015), enhance competence of oocytes matured in vitro to form blastocysts after fertilization (da Silveira et al., 2017) and stimulate proliferation of granulosa cells (Hung et al., 2017).

Here, we verified earlier observations (Rodriques et al., 2015) that follicular fluid can reduce effects of heat shock during maturation on function of the oocyte and tested whether this activity is associated with follicular fluid exosomes. Results support the concept that exosomes present in follicular fluid from small follicles can enhance oocyte function and protect it from deleterious effects of heat shock.

Materials and Methods

Collection of follicular fluid

Follicular fluid was collected by aspiration of ~2-8 mm follicles present on the surface of ovaries obtained from a local abattoir. The cattle used as a source of ovaries were either *Bos taurus* or admixtures of *B. taurus* and *B. indicus*. The ovaries were transported from the abattoir in 0.9% (wt/vol) NaCl containing 100 U/mL penicillin-G and 100 µg/mL streptomycin at 23°C. A total of four batches of follicular fluid (32-50 mL per batch) were collected in March and April 2017

from 48-70 ovaries per batch. Each batch was stored at -80°C until processing for follicular fluid or exosome purification.

Isolation of exosomes

For each batch of follicular fluid, a total of 30 mL was thawed and centrifuged at 1000 x g for 15 min at 4°C and the supernatant fraction was filtered using a 0.2 μm rapid-flow Nalgene filter (Thermo Fisher Scientific, San Jose, CA, USA). The filtered material was used for follicular fluid supplementation and exosome extraction.

For exosome purification, the filtered supernatant fraction was subjected to two rounds of ultracentrifugation at 118,000 x g for 70 min at 4°C using a fixed-angle Ti-70 rotor (Beckman Coulter, Inc., Pasadena, CA, USA). The pellet, which contained exosomes, was resuspended in 200 μL of sterile phosphate buffered saline (PBS) consisting of 1.06 mM potassium phosphate monobasic; 2.97 mM sodium phosphate dibasic and 155 mM NaCl, pH 7.4 and 280 – 315 mOsm/kg). The resuspended pellet was stored at -80°C.

Characterization of exosomes

Concentration and size of exosomes in the pellet were determined by nanoparticle tracking analysis using a NanoSight NS300 (Malvern Instruments, UK) as previously described [20]. The exosomal pellet was diluted 1:1000 (v/v) in PBS before analysis.

Protein concentration was determined using the bicinchoninic acid protein assay (Thermo Fisher) after exosomes were diluted with an equal volume of radioimmunoprecipitation assay buffer (Thermo Fisher) and sonicated twice while on ice for 5-sec each. Protein in the exosomal lysate (50 μg) was subjected to sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting to identify the exosome marker flotillin-1. Procedures were as described previously (Dang et al., 2017) and the antibody was 1 μg/mL of rabbit polyclonal

anti-flotillin-1 antibody (Abcam, Cambridge, MA, USA). The immunogen was a synthetic peptide corresponding to residues 1-100 of human flottilin-1 conjugated to keyhole limpet hemocyanin.

Identification of proteins in each of four preparations of exosomes was determined by mass spectrometry. For each of four samples, 50 µg of total protein were separated by SDS-PAGE usingprecast 4-20% polyacrylamide gradient gels (Bio-Rad, Hercules, CA, USA). Protein bands were visualized by Coomassie labeling. The whole protein lanes were cut and transferred into 1.5 ml conical tube. The sample were destained with 50 mM ammonium bicarbonate and digested with 6 µg/mL modified trypsin (Promega, Madison, WI, USA) at 37°C overnight. Tryptic digests were lyophilized solid phase extraction performed to remove impurities using SOLA SPE cartridge (Thermo Fisher) according to the manufacturer's instruction.

Samples were analyzed using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher) equipped with a Nanospray Flex Ion Source coupled to an EASY-nLC 1200 system (Thermo Fisher). Peptides were eluted using a Acclaim Pepmap 100 precolumn (20 mm×75µm; 3µm-C18; Thermo Fisher) and separated on a PepMap RSLC analytical column (250 mm×75µm; 2µm-C18; Thermo Fisher) at a flow rate at 300 nL/min during a linear gradient from solvent A [0.1% formic acid (v/v)] to 40% solvent B [0.1% formic acid and 80 % acetonitrile (v/v)] over 90 min followed by ramp up to 98% solvent B in 5 min and held for 25 min at a spray voltage of 2.0 kV. Data were acquired by MS1 analysis in the orbitrap (m/z 350 to 1800, resolution 120,000) followed by eight data-dependent MS/MS events with high resolution product ion analysis (isolation window 1.3 Th) performed in the ETD-enabled quadrupole linear ion traps with a fixed scan cycle time of 2 s. The form of MS/MS activation was chosen

depending on the nature of the selected ions using a data-dependent decision tree (Swaney et al., 2008). Doubly charged ions, triply charged ions with m/z to 650, quadruply charged ions with m/z to 900, and quintuply charged ions with m/z to 950 were triggered with either collision induced dissociation (CID) only or electron transfer dissociation (ETD) only. CID was performed at 35% normalized collision energy (NCE), and ETD and CID were carried out with a maximum injection time of 35 ms. A target value of 10,000 was selected for MS2 automatic gain control, and precursor ions were dynamically excluded for 30 s.

Tandem mass spectra were extracted by Proteome Discoverer (version 2.2; Thermo Fisher Scientific, San Jose, CA, USA). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (version 2.4.1; Matrix Science, London, UK). Mascot was set up to search the Uniprot *Bos taurus* database (downloaded on Feb. 13 2018, 32231 entries; http://www.uniprot.org/uniprot/?query=bos+taurus&sort=score) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Gln->pyro-Glu of the n-terminus, deamidated of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications. Scaffold (version 4.2.1; Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and

149 could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles
150 of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.
151 To gain insights into the biological processes that proteins found in the exosomes
152 participate in, gene ontology analysis was performed using UniProtKB (UnitProt Consortium,
153 2017). A total of 71 proteins was analysed. Proteins were chosen because the spectral count was
154 $\geq$ 20 for each of the four samples analysed.
155 *In vitro maturation, fertilization and embryo culture*
156 Ovaries used to collect oocytes were similar to those described above. Oocytes were collected by
157 bisecting follicles ~2-8 mm in diameter with a scalpel and rinsing the ovaries through a solution
158 of BoviPRO^{TM} oocyte washing medium (MOFA Global, Verona, WI, USA). Cumulus-oocyte
159 complexes (COC) were selected under a dissecting microscope, washed 3 times in oocyte
160 washing medium and transferred to 50 µL microdrops of oocyte maturation medium (BO IVM,
161 IVF Biosciences, Falmouth, United Kingdom) overlaid with mineral oil (Sigma-Aldrich, St.
162 Louis, MO, USA). The COC were matured for 22 h at 38.5°C in an atmosphere of 5% (v/v) CO_2
163 in humidified air. Other media used were prepared as described elsewhere (Ortega et al., 2017).
164 After maturation, COC were washed 3 times in HEPES-TALP and placed in groups of up
165 to 65 in a 35 mm dish of 1.7 mL of IVF-TALP to which 80 µL of a solution of PHE (0.5 mM
166 penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine) and 120 µl of sperm (final
167 concentration, 1 x 10^6 cells/mL) were placed. For each replicate, sperm were derived from straws
168 of frozen-thawed semen from 3 individual bulls. Sperm were purified in a gradient of PureSperm
169 (Nidacon, Mölndal, Sweden) for 10 min at 1000 x g and washed in HEPES-TALP for 5 min at
170 200 x g. A different assortment of bulls were used for each replicate. Gametes were co-incubated
171 for 18 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air.

Oocytes exposed to sperm were denuded of cumulus cells in a vortex with 1.4 U/mL hyaluronidase in 0.9% (wt/vol) NaCl, washed 3 times in HEPES-TALP and transferred in groups of 30 to 50 µL microdrops of synthetic oviduct fluid bovine embryo 2 overlaid with oil. Embryos were cultured in an atmosphere of 5% CO_2, 5% O_2 and the balance N_2 in humidified air. The proportion of oocytes that cleaved was determined at 3 d after insemination and blastocyst development was determined at 7.5 d after insemination.

Experiments with heat shock

Three experiments were conducted to evaluate whether follicular fluid or follicular fluid exosomes would reduce detrimental effects of heat shock on COC. In the first experiment, COC were assigned randomly to one of four treatments arranged in a 2 x 2 factorial design with main effects of incubation temperature during maturation (38.5 vs 41°C) and concentration of follicular fluid in the maturation medium [0 or 10%, (v/v)]. The COC were matured at either 38.5°C for 22 h or, for the heat shock treatment, for 41°C for 14 h followed by 8 h at 38.5°C. The COC were cultured in microdrops that consisted of either 50 µl maturation medium or 45 µL maturation medium and 5 µL follicular fluid. Thereafter, fertilization and embryo culture proceeded as described above to determine cleavage and blastocyst development. The experiment was replicated 4 times, using a total of 210-222/oocytes per treatment.

The second and third experiments were conducted similarly to the first except that treatments were arranged in a 2 x 3 factorial design with main effects of temperature (38.5 or 41°C as described earlier) and with three culture media – vehicle (PBS), 10% (v/v) follicular fluid, or exosomes (final concentration of 16 x 10^9 particles/mL). A total of 4 different samples of follicular fluid and 4 preparations of exosomes were used in each experiment. The exosomes

were prepared by adding 2.3-4.70 µL exosomes to 400 µL maturation medium. The vehicle control was prepared by adding a similar volume of PBS to 400 µL maturation medium.

For the second experiment, expansion of COC was assessed before maturation and again at 22 h after maturation. Digital images of each maturation drop were obtained with an EVOS XL Core Imaging System microscope (ThermoScientific, Maltham, MA, USA; 4x magnification). Images were analysed using Image J v 1.43 software (U.S. National Institutes of Health, Bethesda, MD, USA) to determine the surface area of the COC. Separate drops of COC were evaluated before and after maturation and average surface area of COC in a drop calculated. The increase in mean surface area of COCs after maturation was determined by subtracting mean surface area before maturation from mean surface area after maturation. The experiment was replicated 5 times using a total of 119 - 122 COC/treatment.

For the third experiment, COC were fertilized and the resultant embryos cultured as described for the first experiment to determine cleavage and blastocyst development. The experiment was replicated 6 times using 244-286 oocytes/treatment. Blastocysts from the third experiment were collected for analysis of transcript abundance by quantitative polymerase chain reaction (PCR). A total of 3 pools of 10 blastocysts/ pool were collected for each treatment. Blastocysts were treated with 0.1% (wt/vol) proteinase from *Streptococcus griseus* (Roche, Nutley NJ, USA) to remove the zona pellucida. Blastocysts were then washed in Dulbecco's phosphate buffered saline (DPBS) containing 1% (w/v) polyvinylpyrrolidone (PVP) (DPBS-PVP), frozen in liquid nitrogen and stored at -80°C. RNA was extracted with PicoPure RNA isolation kit (ThermScientific) using the manufacturer's instructions. For PCR, samples were thawed, treated with DNase I and subjected to reverse transcription with High Capacity cDNA RT kit (Qiagen). PCR was performed using a CFX96 Real-Time PCR Detection System (Bio-

Rad, Hercules, CA, USA) and the SYBR green mix. Each reaction contained 0.75 µL forward primer (10 mM), 0.75 µL reverse primer (10 mM), 12.5 µL SYBR Green Mix (ThermoScientific), and 8.5 µL diethyl pyrocarbonate water. The volume of 18.8 µL reaction was mixed with 1.2 µL of cDNA sample and samples were run in duplicate. Amplification conditions were: 95°C for 30 sec, 40 cycles at 95°C for 5 sec, 60°C for 5 sec, and 1 cycle of melt curve analysis at 65–95°C in increments of 0.5°C every 5 sec. Primers used were described elsewhere for *YWHAZ* (Gooessens et al., 2005), *NANOG* and *SOX2* (Ozawa et al., 2012) and *GAPDH* (Kannampuzha-Francis et al., 2017).

Uptake of exosomes by oocytes and cumulus cells

Exosomes extracted from a single preparation of follicular fluid (20 µL) were incubated with 10 µM BODIPY TR (Invitrogen, Waltham, MA, USA) for 20 min at 37°C. The excess dye was removed by centrifugation at 750 x g for 2 min in an Exosome Spin Column (Invitrogen). Labelled exosomes (16 x 10^9 particles/mL) were added in 5 µL to groups of 30 COC in 45 µL maturation medium using procedures described above. COC were then matured for up to 22 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. COC were removed from culture after 0, 0.5, 1, 14 and 22 h. Oocytes and cumulus cells were separated mechanically by pipetting in PBS-PVP and then fixed in 4% (wt/vol) formaldehyde in PBS-PVP for 15 min and labelled with 1 µg/mL Hoechst 33342 in PBS-PVP for 15 min. Specimens were evaluated for labelling using an Olympus IX2-DSU spinning disk confocal fluorescent microscope using DAPI (Ex 387/11, Em 440/40) and Texas Red (Ex 562/40, Em 624/40) filters. Z-stack images were generated by collecting a series of images at 0.75 µm intervals for cumulus cells and 3 µm for oocytes. A total of 5 oocytes and 5 sets of cumulus cells were examined at each time point.

Statistical analysis

Data for percent cleavage, percent oocytes exposed to sperm that developed to the blastocyst stage, percent of cleaved embryos that developed to the blastocyst stage, area of the COC and gene expression were calculated for each replicate. PCR data were analysed using the $2^{\Delta\Delta Ct}$ method. Fold change was calculated relative to the value for the vehicle-38.5°C group. The value for housekeeping genes was the geometric mean of the Ct for *GAPDH* and *YWHAZ*. Data were then subjected to least-squares analysis of variance using the General Linear Models procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The mathematical model included effects of maturation temperature, treatment, replicate and all interactions. For experiments with 3 treatments (vehicle, follicular fluid and exosomes), orthogonal contrasts were used to partition variance for effects of treatment and the temperature x treatment interaction. The contrasts were 1) vehicle vs follicular fluid + exosomes and 2) follicular fluid vs exosomes. In addition, the pdiff mean separation test was used to determine effects of heat shock for each individual treatment.

Results

The effect of follicular fluid on developmental competence of oocytes

Results are shown in Fig. 1. Exposure of COC to a heat shock of 41°C for the first 14 h of maturation reduced cleavage rate (P=0.019) and the percent of oocytes (P=0.0015) and cleaved embryos (P=0.0043) that developed to the blastocyst stage. There was no effect of follicular fluid on cleavage rate (Fig. 1A) but addition of follicular fluid to a final concentration of 10% increased development to the blastocyst stage, whether expressed as percent oocytes becoming blastocysts (Fig. 1B; P=0.0058) or percent cleaved embryos becoming blastocyst (Fig. 1C; P=0.0234). The temperature by heat shock interaction approached significance for percent

oocytes becoming blastocysts (P=0.0788). The interaction resulted because the reduction in development caused by heat shock was greater for oocytes cultured with vehicle than for oocytes cultured with follicular fluid.

Characterization of exosomes

Nanoparticle tracking analysis was performed on 4 separate exosome samples using a NanoSight NS300 system. As shown for the representative analysis of a sample (Fig. 3A), the majority of particles present were less than 300 nm in diameter and can be classified as exosomes. The concentration of exosomes for each preparation varied between 1 and $5x10^{11}$ particles/mL. Western blot analysis confirmed that the exosomal marker flotillin-1 was detected in each of the four exosomal preparations (Fig. 3B).

Identification of proteins in each of four preparations of exosomes was determined by mass spectrometry. The total number of proteins with at least 1 spectral count ranged from 869 to 1059. The number of proteins found in each sample with a minimum of 20 spectral counts was 77, 94, 82 and 83 for each individual sample. Information on proteins detected in each of the four samples of exosomes is presented in Supplementary File S1.

There were 71 proteins with spectral count $\geq$ 20 that were present in all four samples; the identity and mean spectral count for these proteins as well as gene ontologies are listed in Supplemental File S2. The biological process ontologies containing the most proteins were *cellular process* and *biological regulation*, the cellular component ontologies containing the most proteins were *extracellular region part* and *cell part* and the molecular function containing the most proteins was *binding*. Many proteins found in plasma were identified including several serine proteinase inhibitors, complement proteins, fibrinogens, inter-α-trypsin inhibitor heavy chains, and immunoglobulin proteins as well as α2-macroglobulin, apolipoprotein A1, albumin,

serotransferrin, vitamin D binding protein and vitronectin. Three heat shock proteins were present (heat shock protein 27 kDa protein 1, heat shock cognate 71 kDa protein and clusterin). Proteins involved in cytoskeleton that were identified included several tubulin proteins and gelsolin. The trafficking molecule clathrin heavy chain was identified as was the transmembrane protein prostaglandin F2 receptor inhibitor. Proteins involved in cell adhesion included vitronectin and lactadherin.

The effect of follicular fluid exosomes on cumulus cell expansion of bovine oocytes exposed to heat-shocked

Exposure of COC to heat shock reduced (P=0.001) cumulus cell expansion (Fig. 3). Addition of either follicular fluid or exosomes increased cumulus cell expansion compared to vehicle (P=0.053). There was, however, no interaction between temperature and treatment.

The effect of follicular fluid and follicular fluid exosomes to reduce effects of heat shock on developmental competence of oocytes

As shown in Fig. 4A, cleavage rate was reduced by heat shock (P=0.0149) and was higher for oocytes cultured with either follicular fluid or exosomes than for oocytes cultured with vehicle (vehicle vs follicular fluid + exosomes, P=0.063). Moreover, the effect of heat shock was reduced by both follicular fluid and exosomes (temperature x vehicle vs follicular fluid + exosomes, P=0.0098).

Heat shock also reduced development to the blastocyst stage, whether expressed as percent of oocytes becoming blastocysts (Fig. 4B; P=0.048) or percent of cleaved oocytes (Fig. 4C; P=0.062). Overall, development of oocytes (P=0.019) or cleaved oocytes (P=0.052) to the blastocyst stage was higher for oocytes treated with follicular fluid or exosomes than oocytes treated with vehicle. Interactions between temperature and treatment were not significant but

analysis of differences between individual means using pdiff indicated that heat shock reduced the percent of oocytes becoming blastocysts for oocytes treated with vehicle (P=0.0068) and follicular fluid (P=0.080) but not for oocytes treated with exosomes (Fig. 4B). Similar results were found for percent of cleaved embryos becoming blastocysts. Heat shock reduced development for oocytes treated with vehicle (P=0.014) but not for oocytes treated with follicular fluid or exosomes.

Expression of *NANOG* and *SOX2* in blastocysts derived from oocytes of each treatment was examined to determine effects of temperature and treatment on selected genes important for pluripotency of the blastocyst. Overall, there was no effect of temperature or treatment on transcript abundance of *NANOG* (Fig. 5A). There was, however, an interaction between temperature and the comparison of vehicle vs follicular fluid + exosomes (P=0.058). This interaction resulted because heat shock reduced *NANOG* expression for blastocysts from oocytes treated with exosomes only (P=0.060). There were no effects of temperature or treatment on expression of *SOX2* (Fig. 5B).

Exosome uptake

Cumulus-oocytes complexes were incubated with labelled exosomes for up to 22 h during oocyte maturation to determine whether cumulus cells or oocytes take up exosomes (Fig. 6). There was no specific labelling of cumulus cells with exosomes after 0.5 h of incubation (note the non-specific red fluorescence in panel A than it similar in intensity to fluorescence in cumulus cells incubated without labelled exosomes in panel E). Thereafter, however, labelled spherical structures were associated with cumulus cells, at 1 h (Fig. 6B), 14 h (Fig. 6C) and 22 h (Fig. 6D). In contrast, there was no association of labelled exosomes with oocytes at any time (Fig. 6F). Further analysis of labelling of individual cumulus cell by tracing labelling through Z-stack

sections indicated that labelled exosomes could be identified inside the cell including adjacent to the nucleus (Fig. 7).

Discussion

Communication between cellular constituents of the ovarian follicle and between the follicle and other organs, is essential for the follicle to achieve its twin goals of steroid hormone secretion and production of an oocyte capable of fertilization and supporting subsequent embryonic development (Monniaux, 2016; Russell et al., 2016; LaVoie, 2017). It has been shown previously that addition of follicular fluid to oocyte maturation medium can increase cumulus cell expansion, reduce cumulus cell apoptosis, increase competence of the oocytes to be fertilized and develop to the blastocyst stage, and protect the oocyte from heat shock (Rodrigues et al., 2015; Grupen and Armstrong, 2010; Somfai et al., 2012). Similar effects of follicular fluid were observed in present study. Moreover, it is likely that this property of follicular fluid resides, at least in part, in the exosomal fraction of the fluid. In particular, exosomes isolated from follicular fluid from small follicles acted on the COC during oocyte maturation to enhance cumulus cell expansion and increase competence of the oocyte to cleave after fertilization and to support development to the blastocyst stage. Moreover, exosomes could reduce consequences of exposure of the COC to heat shock with respect to effects on cleavage rate and development of embryos to the blastocyst stage. Taken together, results support the idea that exosomes in follicular fluid play important roles during oocyte maturation to enhance oocyte function and protect it from stress. One practical implication is that exosomes may prove useful in enhancing outcomes of embryo production systems in vitro.

It is likely that exosomes affect oocyte function not by acting directly on the oocyte but rather by affecting function of associated cumulus cells. Indeed, as has been reported previously

(Hung et al., 2015), cumulus cells but not oocytes took up labelled exosomes derived from follicular fluid. The uptake of exosomes is performed by endocytosis or membrane fusion (Mulcahy et al., 2014) and the endocytic capacity of the oocyte is reduced after zona pellucida formation (Fair et al., 1997). Additional evidence that exosomes were acting on cumulus cells was the finding that exosomes increased cumulus cell expansion during maturation. Similar effects of follicular-fluid derived exosomes on cumulus cell expansion have been reported in cattle (Hung et al., 2015) and pigs (Matsuno et al., 2017).

It is likely that the effects of exosomes on oocyte competence are not due directly to the effect on cumulus expansion. In mice, genetic models in which cumulus expansion is compromised is associated with normal fertilization and embryonic development (Ploutarchou et al., 2015; Bertolin et al., 2017). Other reported effects of exosomes on cumulus cell function during maturation include alterations in gene expression (Hung et al., 2015) and it is possible that regulation of cumulus gene expression by exosomes results in downstream improvements in oocyte function.

In addition to improving cumulus cell expansion and oocyte competence for fertilization, treatment with exosomes increased the resistance of the COC to heat shock. In particular, the negative effects of heat shock on cleavage rate and development to the blastocyst stage, although not cumulus cell expansion, were reduced in COC cultured with either follicular fluid or exosomes. Such a result points to a physiological role for exosomes in follicular fluid to protect the oocyte during maturation from disruption by elevated body temperature (Putney et al., 1988).

The cytoprotective effects of follicular fluid exosomes are probably not limited to heat shock but extend to other stresses as well. This conclusion is based on recent results regarding oxidative stress (Guay and Regazzi, 2017). In particular, bovine granulosa cells exposed to

hydrogen peroxide released exosomes into the culture medium that were enriched for mRNA encoding for genes involved in antioxidant defense such as *NRF2, CAT* and *TXN1*. Incubation of granulosa cells with exosomes secreted from cells subjected to oxidative stress resulted in uptake of the exosomes, increased transcript abundance for *NRF2*, *PRDX1, CAT and TXN1*, and reduced effects of hydrogen peroxide on reactive oxygen species content, mitochondrial activity, cell cycle and cell proliferation.

Exosomes have been implicated in communication between organs through secretion into blood (Guay and Regazzi, 2017; Fleshner and Crane, 2017) and some follicular fluid exosomes are probably produced outside the follicle. However, the composition of exosomes in follicular fluid differs from exosomes in blood in women (Santonocito et al., 2014) and local sources of exosomes include granulosa cells (Saeed-Zidane et al., 2017; Vashisht et al., 2018). The oocyte has also been reported to produce exosomes (Barraud-Lange et al., 2012; Benammar et al., 2017) although it is not known whether oocyte-derived exosomes cross the zona pellucida.

The molecular pathways by which exosomes affect COC to improve oocyte competence and thermotolerance remain to be determined. Exosomes can transport mRNA, microRNA, long non-coding RNAs, mitochondrial DNA, proteins and phospholipids (Choi et al., 2013; DiPietro, 2016), any of which could conceivably affect cumulus cell function. There is evidence for exosome-mediated delivery of microRNA (Sohel et al., 2013) and mRNA (Guay and Regazzi, 2017) to bovine granulosa cells and evidence that exosome-delivered microRNA can change abundance of mRNA targeted by the microRNA (Sohel et al., 2013). Exosome-derived microRNA are also involved in mediating regulation of granulosa cell proliferation (Hung et al., 2017). Among the proteins present in exosomes were two heat shock proteins that confer

400 thermotolerance – heat shock protein 27 (carper et al., 1997; Doerwald et al., 2003; Shi et al.,
401 2011) and heat shock cognate 71 kDa protein (Ciavarra et al., 1994).
402 Overall, the protein composition of the exosomes isolated from bovine follicular fluid for
403 the current experiments was comparable to that of exosomes isolated from fluid of dominant
404 follicles from the mare (da Silveira et al., 2012). Most similar was a predominance of proteins
405 characteristic of plasma and other extracellular fluids. In addition, several intracellular or
406 membrane proteins found in exosomes from other fluids were identified in follicular fluid
407 exosomes. These included clathrin heavy chain, heat shock cognate 71 kDa, gelsolin,
408 lactadherin, and several tubulin proteins (Hegmans et al., 2004; Conde-Vancells et al., 2008; da
409 Silveira et al., 2012; Dang et al., 2017).
410 The expression of *NANOG* and *SOX2* in blastocysts was measured to ascertain whether
411 blastocyst characteristics were altered by treatment of the oocytes from which they were derived.
412 There were no consistent effects of follicular fluid, exosomes or heat shock on expression of
413 either of these two pluripotency genes. The lack of effect of oocyte heat shock on gene
414 expression of the resultant blastocysts is consistent with studies showing no difference in
415 blastocyst cell number between blastocysts derived from control or heat-shocked oocytes (Roth
416 and Hansen, 2004ab; Lima et al., 2017; Ascari et al., 2017) although there is at least one report
417 that cell number of blastocysts is reduced by oocyte heat shock (Rodrigues et al., 2016).
418 The current experiments were performed using follicular fluid from small follicles.
419 However, the events studied here occur during oocyte maturation when the COC is enclosed in a
420 large preovulatory follicle. It is possible that the exosomes exerting effects seen here are absent
421 or present in reduced amounts in follicular fluid from large preovulatory follicles. Indeed,
422 concentration of exosomes in follicular fluid of the cow decreases as follicles increase in size and

423 there are concomitant changes in the exosomal microRNA content (Navakanitworakul et al.,
424 2016). Moreover, exosomes from small follicles are taken up more readily by granulosa cells
425 than exosomes from large follicles (Hung et al., 2017).

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429 these experiments.

430 **Conflict of Interest**

431 The authors declare no conflicts of interest.

432

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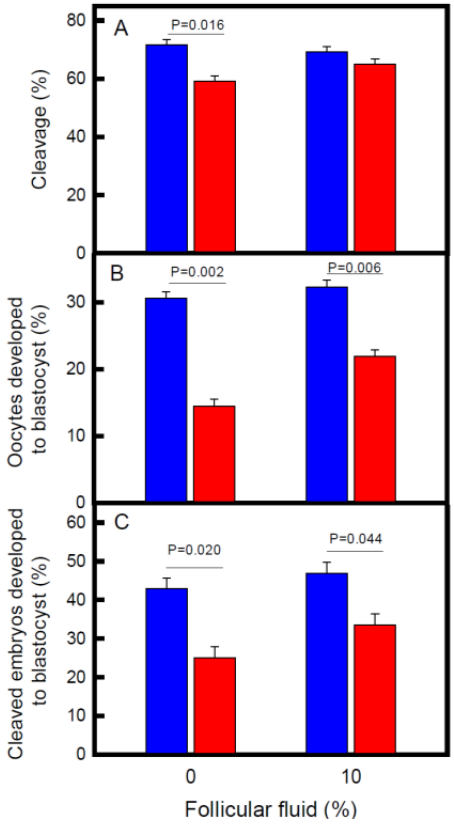
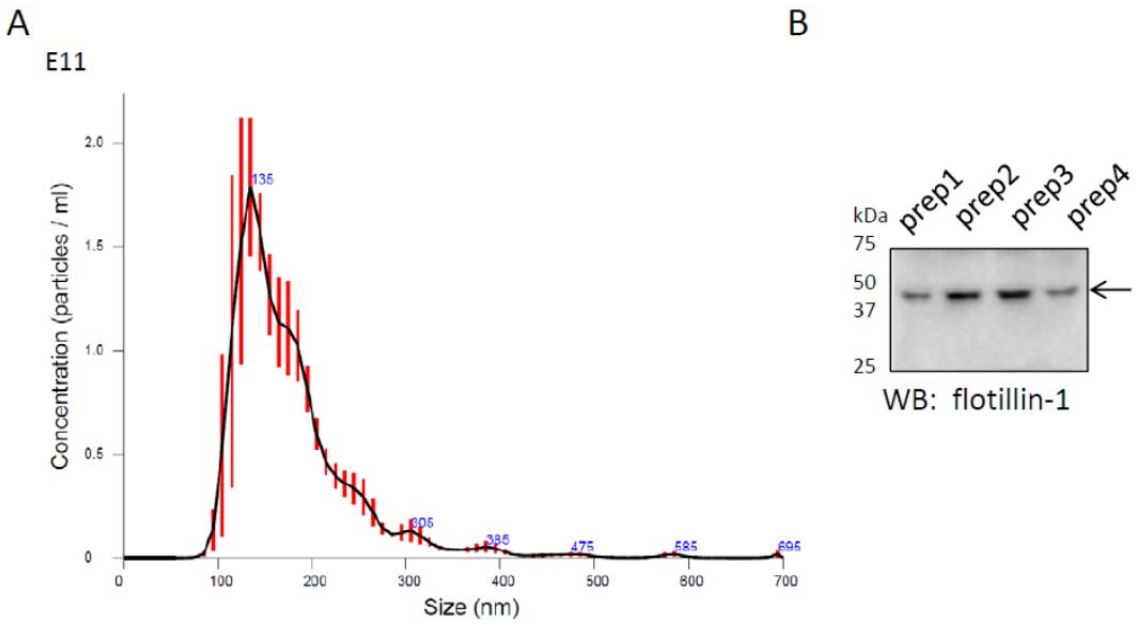


Fig. 1. The effect of follicular fluid on developmental competence of oocytes matured at 38.5 (blue) or 41°C (red). After maturation, oocytes were fertilized and allowed to develop until day 7.5 after insemination. Results are least-squares means ± SEM of 4 replicates using 210-222 oocytes/treatment. Lines over pairs of bars represent effects of heat shock for an individual treatment – temperature combination. In addition, analysis of variance indicated that maturation at 41°C reduced cleavage rate (panel A; P=0.019) and the percent of oocytes (panel B; P=0.0015) and cleaved embryos (panel C; P=0.0043) that developed to the blastocyst stage. Addition of 10% follicular fluid increased development to the blastocyst stage, whether expressed as percent oocytes becoming blastocysts (panel B; P=0.0058) or percent cleaved embryos becoming blastocyst (Fig. 1C; P=0.0234). The temperature by heat shock interaction approached significance for percent oocytes becoming blastocysts (P=0.0788). There was a temperature effect ($P < 0.05$) on the percentage of embryos cleaved.



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621

622 **Fig. 2.** Characterization of follicular fluid exosomes. A. Representative analysis of an exosomal
623 preparations by Nanosight (n=4) showing the concentration of follicular fluid exosomes in
624 particles per mL and the diameter of the exosomes in nanometers (nm). The sample was
625 analyzed in triplicate and red lines are standard error bars. Peaks are indicated by blue numbers.
626 B. Western blot analysis of the four exosome preparations (prep1-prep4) used in the
627 experiments.

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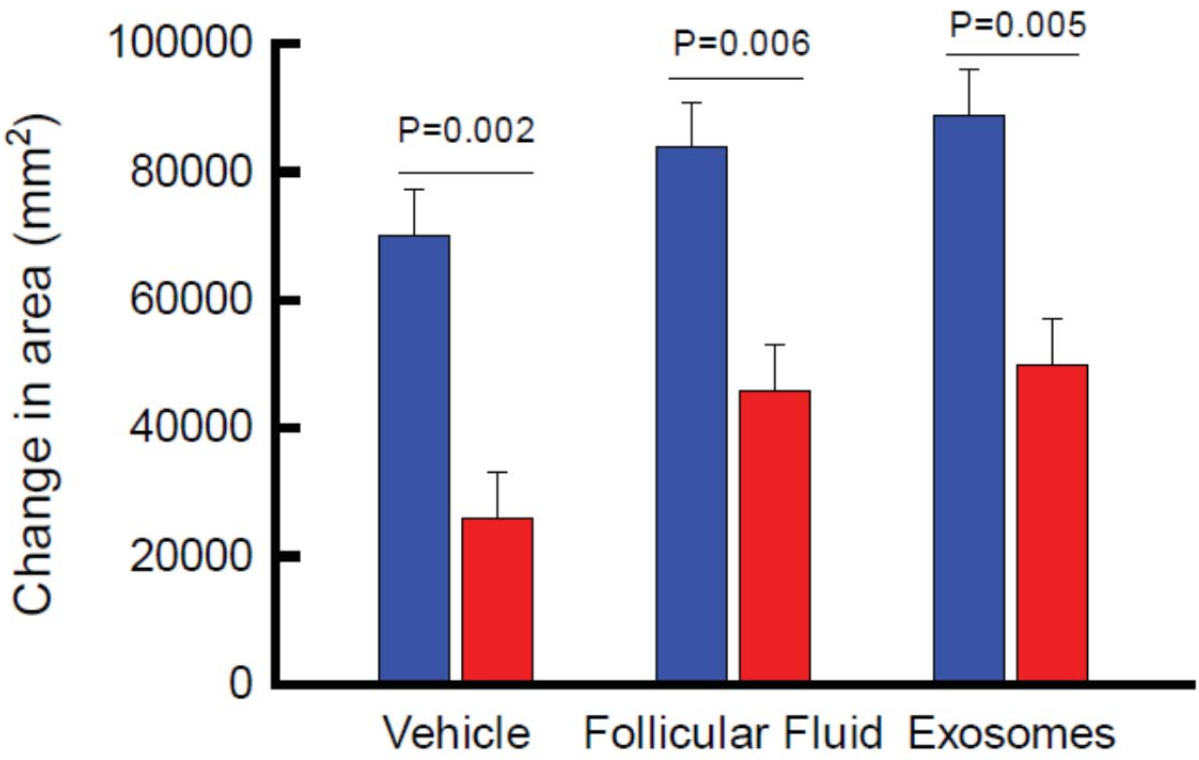
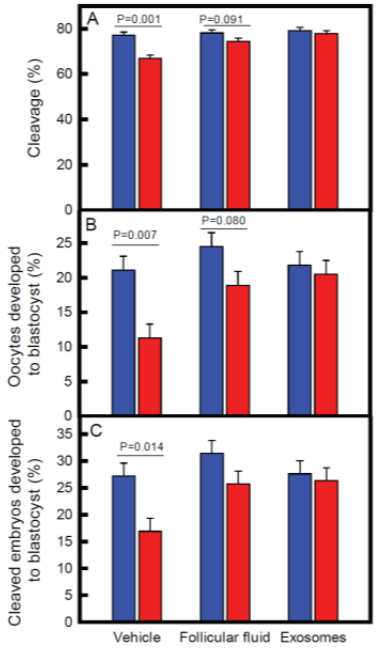


Fig. 3. The effect of follicular fluid and exosomes added during maturation on cumulus expansion in cumulus-oocyte complexes (COC) matured at 38.5 (blue) or 41°C (red). The increase in mean surface area of COCs after maturation was determined by subtracting mean surface area before maturation from mean surface area after maturation. The experiment was replicated 5 times using a total of 119 - 122 cumulus-oocyte complexes/treatment. Results are least-squares means ± SEM. Lines over pairs of bars represent effects of heat shock for an individual treatment – temperature combination. In addition, analysis of variance indicated that maturation at 41°C reduced (P=0.001) cumulus cell expansion. Addition of either follicular fluid or exosomes increased cumulus cell expansion compared to vehicle (P=0.053). There was, however, no interaction between temperature and treatment.



641

642 **Fig. 4.** The effect of follicular fluid and exosomes on developmental competence of oocytes
643 matured at 38.5 (blue) or 41°C (red). After maturation, oocytes were fertilized and allowed to
644 develop until day 7.5 after insemination. Results are least-squares means ± SEM of 6 replicates
645 using 244-286 oocytes/treatment. Lines over pairs of bars represent effects of heat shock for an
646 individual treatment – temperature combination. In addition, analysis of variance indicated the
647 following effects. Cleavage rate (A) was reduced by maturation at 41°C (P=0.0149), was higher
648 for oocytes cultured with either follicular fluid or exosomes than for oocytes cultured with
649 vehicle (vehicle vs follicular fluid + exosomes, P=0.063) and there was an interaction between
650 temperature and the vehicle vs follicular fluid + exosomes comparison (P=0.0098). The percent
651 of oocytes that developed to the blastocyst stage (B) was affected by maturation temperature
652 (P=0.0048) and the comparison of vehicle vs follicular fluid + exosomes (P=0.019). The percent
653 of cleaved embryos that developed to the blastocyst stage was affected by maturation
654 temperature (P=0.062) and the comparison of vehicle vs follicular fluid + exosomes ((P=0.052).

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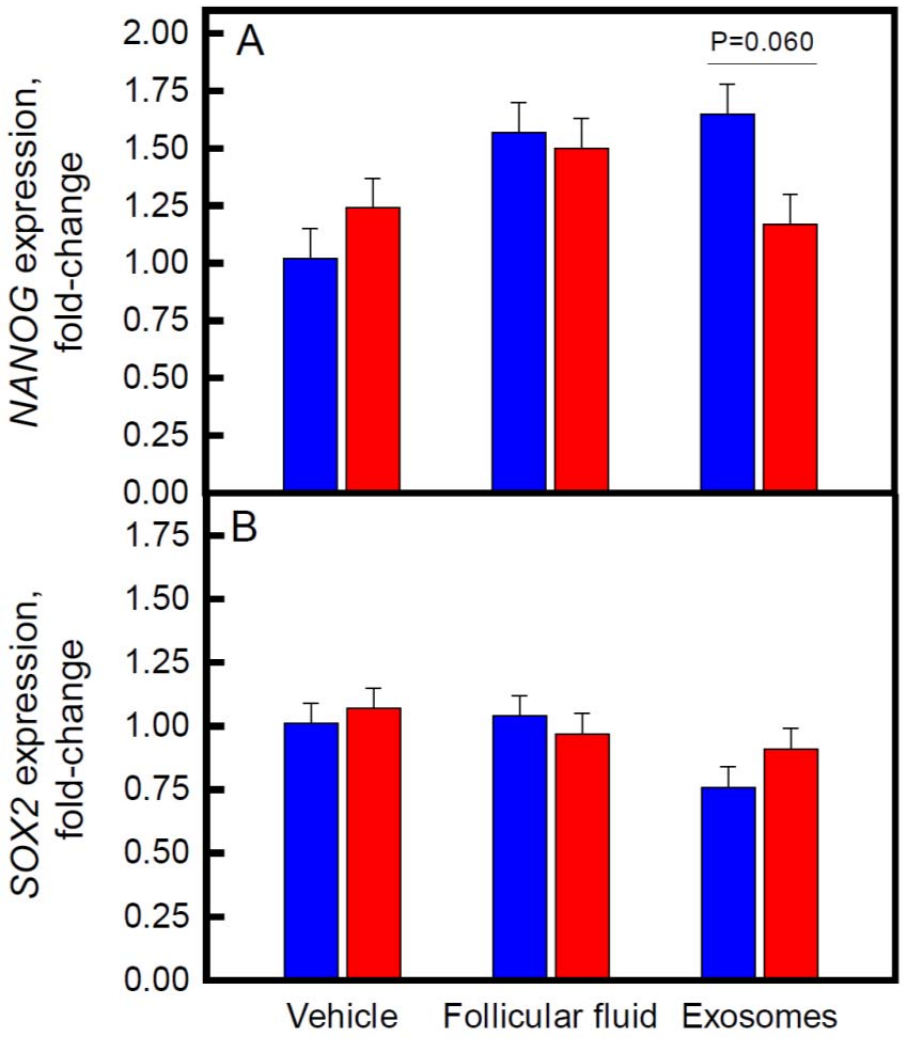
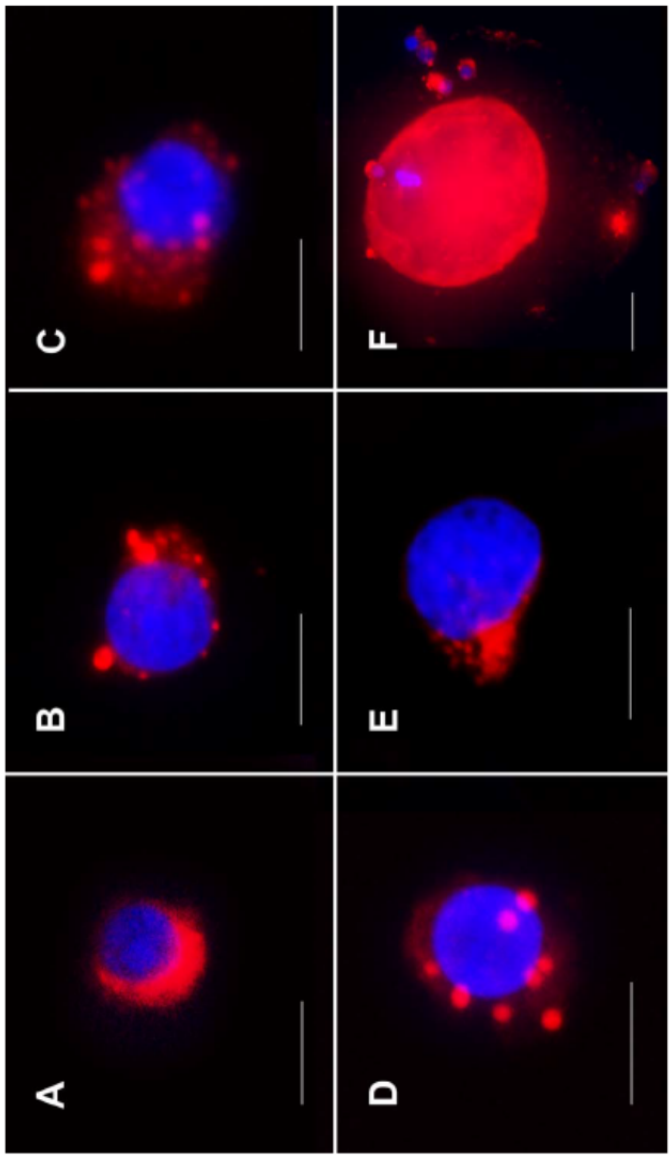


656

657 **Fig. 5.** Blastocyst expression of *NANOG* (A) and *SOX2* (B) for blastocysts derived from oocytes
658 treated with follicular fluid and exosomes during oocytes maturation at 38.5 (blue) or 41°C (red).
659 Results are least-squares means ± SEM of results from a total of 3 pools of 10 blastocysts/pool
660 for each treatment. The lines over one pair of bars represent effects of heat shock for the
661 follicular fluid treated group. In addition, analysis of variance indicated that *NANOG* expression
662 was affected by the interaction between temperature and the comparison of vehicle vs follicular
663 fluid + exosomes (P=0.058). There were no significant effects of expression of *SOX2*.

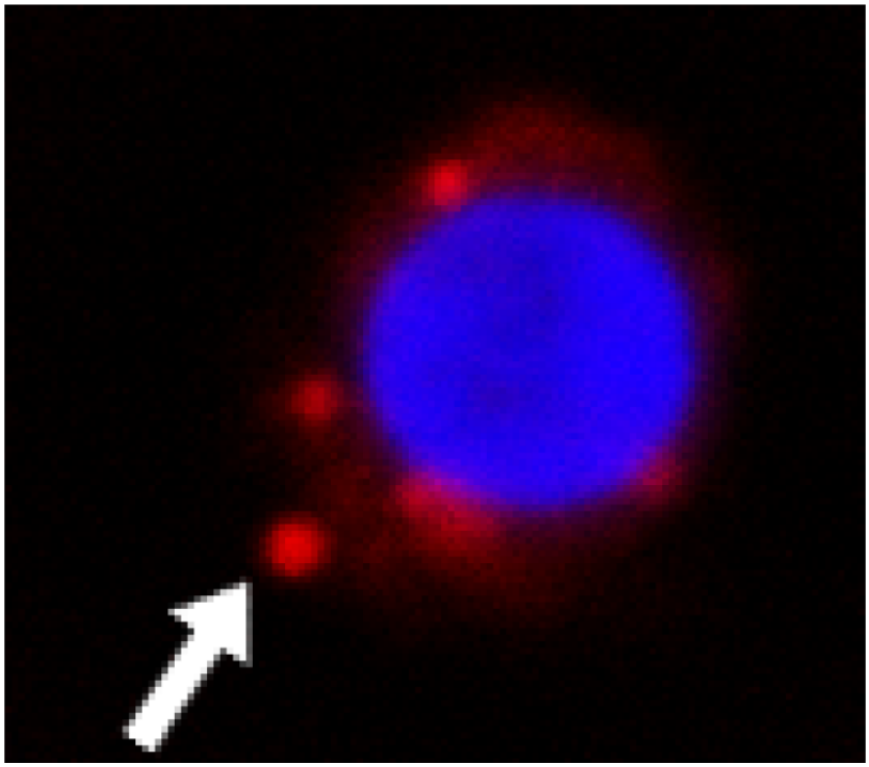
664



665

666 **Fig. 6.** Representative examples of labeling of individual cumulus cells (A-E) and the oocyte (F)
667 after incubation with exosomes labeled with BODIPY TR (red). DNA was also labeled with
668 Hoechst 33342 (blue). Images of cumulus cells were obtained after incubation for 0.5 (A), 1 (B),
669 14 (C) and 22 h (D). Panel E represents a cumulus cell that was incubated without exosomes.
670 The oocyte in panel F was incubated with exosomes for 22 h. White bars represent 10 μM.

671



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673

674 **Fig. 7.** Cross sections of a Z-stacked image (0.75 μm) of a single cumulus cell incubated for 22
675 h with exosomes labeled with BODIPY TR (red). DNA was also labeled with Hoechst 33342
676 (blue). Arrows indicate individual exosomes. White bars represent 10 μM.

677

678 **Supplemental File S1.** Spectral counts for individual peptides and proteins identified in four
679 samples of exosomes from follicular fluid.
680 **Supplemental File S2.** List of most abundant proteins in exosomes isolated from follicular fluid
681 (tab 1) and their classification by gene ontology terms (tab 2).